Jc630 U.S. PTO
02/01/99

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

A/RE

In re Reissue Patent Application of: Mark Lyte
Title: COMPOUNDS FOR MODULATING GROWTH OF INFECTIOUS AGENTS
Attorney Docket No.: 933.001USR

Jc135 U.S. PTO 09/241825 02/01/99

REISSUE PATENT APPLICATION TRANSMITTAL

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

We are transmitting herewith the following attached items and information (as indicated with an "X"):

X Application for **REISSUE** of U.S. Utility Patent No. 5,629,349 (Mark Lyte) comprising:
- X Communication Re: Transmittal of Reissue Application (2 pgs).
- X Copy of the entire specification of the patent (16 columns, including claims numbered 1 through 2 and an abstract).
- X Copy of the printed drawings of the patent (sheets 1 through 16).
- X Declaration [of Inventor] for Broadening Reissue Application (10 pgs).
- X Offer to Surrender Original Patent (1 pg).
- X Assent by Assignee and Power of Attorney (2 pgs).
- X Request to Transfer Drawings (1 pg).
- X Check in the amount of $476.00 to pay the filing fee.

X Information Disclosure Statement (4 pgs), Form 1449 (1 pg), and copies of cited references (6).
X Claims Amendment and Discussion (10 pgs).
X Reassignment Agreement (1 pg), recordation cover sheet, and check in the amount of $40.00 to pay the recordation fee.
X Contribution Agreement (2 pg), recordation cover sheet, and check in the amount of $40.00 to pay the recordation fee.
X Return postcard.

The filing fee has been calculated below as follows:

	No. Filed	No. Extra	Rate	Fee
TOTAL CLAIMS	22 - 20 =	2	x 9 =	$18.00
INDEPENDENT CLAIMS	5 - 3 =	2	x 39 =	$78.00
[ ] MULTIPLE DEPENDENT CLAIMS PRESENTED				$0.00
BASIC FEE				$380.00
TOTAL				$476.00

Please charge any additional required fees or credit overpayment to Deposit Account No. 19-0743.

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.
P.O. Box 2938, Minneapolis, MN 55402 (612-373-6900)

Customer Number 21186

By: [signature]
Atty: Mark A. Litman
Reg. No. 26,390

"Express Mail" mailing label number: EM287852441US Date of Deposit: February 1, 1999
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

By: Chris Hammond Signature: Chris Hammond

REISSUE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): MARK LYTE

PATENT NO.: 5,629,349

ISSUED: MAY 13, 1997

TITLE: COMPOUNDS FOR MODULATING GROWTH OF INFECTIOUS AGENTS

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Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION FOR BROADENING REISSUE APPLICATION

Sir:

I, Mark Lyte, declare as follows:

STATEMENTS SATISFYING 37 C.F.R. §1.63

I believe that Mark Lyte, 4077 Deerwood Trail, Eagan, Minnesota, 55122, United States, was the original, sole and first inventor of the subject matter which is claimed in U.S. Patent No. 5,629,349 (hereinafter "the LYTE patent") and for which additional claims 3 through 20 are hereby presented in the filing of a Reissue Application (Serial Number 09/---,---, hereinafter the "Reissue Application"), and for which additional claims 3-22 have been submitted for examination in the original filing of the Reissue Application.

I was the owner of the entire right, title and interest in and to the LYTE patent, by virtue of a reassignment from Mankato State University to Mark Lyte, and assigned my entire right, title, and interest to BioNutrix, LLC, in a Contribution Agreement, both title documents being filed with the U.S. Patent and Trademark Office contemporaneously with the filing of this Reissue Application.

I have reviewed and understand the contents of the LYTE patent and the claims submitted to the Patent and Trademark Office upon filing of the Reissue Application.

I hereby offer to surrender U.S. Patent No. 5,629,349 upon granting of the Reissue Application as a Reissue Patent.

STATEMENT SATISFYING 37 C.F.R. §1.175(a)(1)

U.S. Patent 5,629,349 is partly inoperative because the claims include more limitations than were necessary to define over the prior art and are, therefore, unnecessarily narrow.

STATEMENT SATISFYING 37 C.F.R. §1.175(a)(3)

New claims 2 through 22 are being added to the present application, the Reissue Application. The new claims have been added because the claims of the LYTE patent fail to provide an appropriate scope of protection. Accordingly, the new claims are submitted to achieve the protection to which the patent owner is entitled.

Claims 3 through 14

Claims 1 and 2, the only claims issued in the LYTE patent, are overly narrow. Each of claims 1 and 2 fail to claim the broadest protection to which the patent owner is entitled. The errors in the original claims arose approximately December 17-19, 1992, as a result of the applicant's failure to appreciate the scope of the protection to which he was entitled, and the filing of a response with claims that were too narrow and which did not reflect the actual scope of protection to which Applicant was entitled. The errors were, in part, discovered during the applicant's discussions upon issuance of the Patent on May 13, 1997. After discussions with my present attorney, Mark A. Litman, on July 3, 1998, I came to understand that the original claims in the Original Application had been written too broadly, reciting "living organisms," rather than the bacteria, viruses and other microorganisms to which the invention actually pertained. I never appreciated, understood, nor was informed of the fact that the original breadth with respect to enhancing growth was too broad and that the scope of claims could have been narrowed to avoid the art cited in the Office Action. Although I presented arguments to the previous attorney of record, no arguments or amendments were made of record to attempt to counter a rejection which was clearly in error. The degree of error becomes apparent in the section of this Declaration entitled "Review of the Art Cited in a Rejection by the Patent and Trademark Office."

U.S. PAT. NO. 5,629,349 claim 1	REISSUE CLAIM 3
1. A method of suppressing the growth	3. A method of enhancing the growth
of Gram-positive bacteria in a host medium,	of bacteria or viruses
said host medium being selected from the group consisting of in vitro and cell cultures,	said host medium being selected from the group consisting of in vitro and cell cultures,
said method comprising the introduction of an effective amount of a catecholamine to the host medium to act directly on the growth of Gram-positive bacteria.	said method comprising the introduction of an effective amount of a catecholamine to the host medium to enhance the growth of said bacteria or viruses.

As is evident from the above comparison, original claim 1 is limited to the method when employed to inhibit the growth of Gram-positive bacteria *in vitro* or in a cell culture. That is an extremely narrow process of little commercial utility. The original application as filed on March 6, 1992 clearly identified the scope of the invention as including enhancing the growth of bacteria and viruses *in vitro* and in cell cultures. Therefore, the LYTE patent is believed to be defective. New claim 3 recites no such limitations with respect to inhibiting growth in only Gram-positive bacteria. Accordingly, independent claim 3 is submitted to properly claim the broadest improvement over the methods of the prior art to which the patentee is entitled. The new claim 3 also does not contain the limitation that the catecholamine "act directly on enhancing" as I only became aware in my discussions with new counsel, Mark A. Litman, that the actual mechanisms (e.g., direct activity) do not have to be recited in claims. The direct activity is recited in claim 4, and claims dependent therefrom.

These claims were rejected in an Office Action mailed on September 25, 1992 for the following grounds and reasons:

I. Claims 1-23 were rejected under 35 U.S.C. 102/103 as unpatentable over Dyer et al. Or Moger et al. It was asserted that each of the references teaches the affecting of the growth of a vector or cell culture using a catecholamine. This was asserted to be what the Applicant was claiming, and therefore the claims were asserted to not be patentable.

II. Claims 1-23 were rejected under 35 U.S.C. 112, second paragraph as failing to particularly point out and distinctly claim the invention. Certain terms such as "vectors," "analogs" and "derivatives" were held to be indeterminate.

III. Claims 1-23 were rejected under 35 U.S.C. 102/103 as unpatentable over Kotimchenko et al. or Sumanskii et al. Each of the references was asserted to show the use of a neurotransmitter chemical to affect the growth of "living organisms." No patentable distinction was seen between the process of the references and the process of the claims.

The response to this Office Action (filed on December 21, 1992) filed new claims 24-33. A restriction requirement, and an asserted constructive election was erroneously made, the restriction was made as between:

I. Claims 29-33 drawn to a method of diagnosis and glucose production, these claims being held to have been constructively non-elected since they were held to have been not previously examined and their subject matter is new.

II. Claims 24-25 drawn to methods of suppressing growth.

III. Claims 26-28 methods of suppressing growth with a catecholamine blocker.

The attorney of record canceled claims 29-33 and elected claims 24 and 25 for prosecution on the merits. These claims were rejected, an Amendment after Final Rejection was filed and refused admission by an Advisory Action. The Application was then refiled as a File Wrapper Continuation, with only claims 24-28 present in the Application.

A restriction requirement was then filed between claims 24-25 and 26-28 in an Office Action mailed January 31, 1995. Applicant then elected claims 24-25 for prosecution on the merits. These claims were then rejected under 35 U.S.C. 112, first and second paragraphs. After

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another series of rejections, with only claims 24-28 in the Application, the two claims in the LYTE Patent were issued.

As is evident from the above comparison, original claim 1 of the LYTE Patent is limited to the method when employed to inhibit the growth of Gram-positive bacteria *in vitro* or in a cell culture. That is an extremely narrow process of little commercial utility. The original application as filed on March 6, 1992 clearly identified the scope of the invention as including enhancing the growth of bacteria and viruses *in vitro* and in cell cultures. Therefore, the LYTE patent is believed to be defective. New claim 3 recites no such limitations with respect to inhibiting growth in only Gram-positive bacteria. Accordingly, independent claim 3 is submitted to properly claim the broadest improvement over the methods of the prior art to which the patentee is entitled.

It is important to note that no restriction requirement in the Application filed on June 27, 1994 was ever asserted against the claims presented in the Reissue Application, so there is no applicability of issues found in *In re Orita, Yahagi, and Enomoti*, 193 USPQ 145, where it was held that "Although appellants undoubtedly erred by failing to file a timely divisional application in order to obtain a divisional patent, it does not follow that such error caused the original patent to be 'partially inoperative by reason of the patentee claiming less than he had a right to claim in the patent' as appellants aver in their reissue declaration under 37 CFR 1.175..." It was further stated in *In re Orita* that "...granting reissue claims substantially identical to those non-elected in application I would be ignoring the proper restriction requirement set forth in that application in which appellants acquiesced. Indeed, appellants' misapplication of section 251 would, if permitted, circumvent the copendency requirement of section 120 incorporated by reference in section." The original restriction requirement was against

1) a method of diagnosing the presence of Gram-negative bacteria, including specific physical steps, none of which are recited in the claims of the Reissue Application;

2) a method of producing glucose from a lactose broth, the claim reciting specific physical steps which are not recited in the claims of the Reissue Application;

3) a method for suppressing the growth of Gram-positive bacteria; and

4) a specific method for suppressing the growth of Gram-negative bacteria comprising the introduction of an effective blocker of catecholamine receptor sites of the organisms.

Methods 1), 2), 3) and 4) are clearly outside the scope of the claimed subject matter of the Reissue Application.

In comparing new claim 3 to original claim 1, claim 1 recites the step of "suppressing the growth of Gram-positive bacteria." Claim 3, however, does not include such a limitation, but instead recites "...enhancing the growth of bacteria or viruses.." This claim is not limited to suppression of growth, but only to enhancing of growth. The only actual restriction requirement which occurred in the prosecution of the U.S. Patent Application U.S. Serial No. 08/266,805 filed on June 27, 1994 was between:

I. Claims 24 and 25, drawn to a method of suppressing the growth of Gram-positive organisms with an amount of catecholamine, classified in Class 514, subclass 727.

II. Claims 26-28, drawn to a method of suppressing the growth of Gram-negative organisms by the introduction of an effective blocker of catecholamine receptor sites of the organisms, classified in Class 514, subclass 224.8.

The constructive election against claims 29-33 found in the parent application preceding U.S. Patent Application U.S. Serial No. 08/266,805 was not a proper restriction requirement, and was substantively incorrect even in its substance. In any event, the claims of the Reissue Application were not the subject of restriction requirements in U.S. Patent Application U.S. Serial No. 08/266,805. Therefore the claims submitted in this Reissue Application do not read on any species, elected invention or non-elected invention for which a proper restriction requirement was made.

In comparing new claim 3 to original claim 1, claim 1 recites the step of "suppressing the growth of Gram-positive bacteria." Claim 3, however, does not include such a limitation, but instead recites "...enhancing the growth of bacteria or viruses.." This claim is not limited to suppression of growth, but only to enhancing of growth. The only restriction requirement which occurred in the prosecution of the U.S. Patent Application U.S. Serial No. 08/266,805 filed on June 27, 1994 was between:

I. Claims 24 and 25, drawn to a method of suppressing the growth of Gram-positive organisms with an amount of catecholamine, classified in Class 514, subclass 727.

II. Claims 26-28, drawn to a method of suppressing the growth of Gram-negative organisms by the introduction of an effective blocker of catecholamine receptor sites of the organisms, classified in Class 514, subclass 224.8.

Therefore the claims submitted in this Reissue Application do not read on any species, elected invention or non-elected invention for which a restriction requirement was made.

In a similar comparison, independent method claim 12 is compared to original method claim 1 for purposes of discussion.

U.S. Pat. No. 5,629,349 claim 1	**REISSUE CLAIM 12**
1. A method of suppressing the growth	12. A method for harvesting the by-products of
of Gram-positive bacteria in a host medium,	enhanced growth of bacteria or viruses comprising
said host medium being selected from the group consisting of in vitro and cell cultures,	introducing an effective amount of a catecholamine to an *in vitro* or cell culture host medium to act directly on enhancing the growth of said bacteria or viruses, and
said method comprising the introduction of an effective amount of a catecholamine to the host medium to act directly on the growth of Gram-positive bacteria.	collecting by-products generated by said bacteria or viruses.

As is evident from the above comparison, original claim 1 is limited to the method when employed to inhibit the growth of Gram-positive bacteria. Accordingly, the claims exclude any useful method of increasing the supply of by-products from said bacteria or cell. The new claim 12 submitted in the Reissue Application encompasses this useful method associated with the enhancement of bacteria or virus growth.

Each of reissue claims 4 through 11 and 13-22 depend from either reissue claim 3 or reissue claim 12, respectively. None of reissue claims 4-11 or 13-22 are believed to be literally restricted to the subject matter which was properly restricted, properly non-elected, and

effectively abandoned in the prosecution of U.S. Patent Application U.S. Serial No. 08/266,805 filed on June 27, 1994 which ultimately issued as U.S. Patent No. 5,629,349 (hereinafter, the "Original Application"). Accordingly, reissue claims 4-11 and 13-22 differ from original claims 1 and 2 and are believed to describe subject matter which is properly the subject of a Reissue Application.

STATEMENT SATISFYING 37 C.F.R. §1.175(a)(5)

During prosecution of the original application, I informed the Attorney of record at the time who was prosecuting the Original Application that the rejection over prior art made in the Office Action mailed on September 25, 1992 was completely erroneous, and that there was absolutely no basis for that rejection being applicable against the scope of invention that I had attempted to cover.

The rejections in the Office Action mailed on September 25, 1992 included rejections of all claims (Claims 1-23) under 35 U.S.C. 102/103 as being unpatentable over Dyer et al., Moger et al., Kotimchenko, or Sukmanskii et al. These rejections were clearly explained to the attorney of record as being completely erroneous, at least for the following reasons:

A. The invention intended to be claimed was the enhanced growth of bacteria or viruses by the administration of catecholamines *in vitro* or in cell cultures. The enhanced growth rate was a result of the addition of the catecholamines.

B. Dyer et al. showed the stimulation of androgen production in ovarian cells when cultured in a serum-free medium. The mechanism proposed in the Chem Abstracts article was that the "catecholamine-augmented androgen prodn. provides a direct link between the autonomic nervous system and regulation of ovarian steroid synthesis." That explanation has no logical bearing or relationship to the stimulation in the growth rate of bacteria or viruses, and none was implied by the article. The reference could neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a rejection under 35 U.S.C. 103.

C. Moger et al. teach that catecholamines stimulated androgen production by mouse interstitial cells in primary culture. The Chem Abstract text has no suggestion on the effect of catecholamine with respect to any cell growth, but only on the stimulation of androgen production. Again, that article has no logical bearing or relationship to the stimulation in the

growth rate of bacteria or viruses, and none was implied by the article. The reference could neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a rejection under 35 U.S.C. 103.

D. Khotimchenko describes the effect of adrenotropic substances (including ephedrine and noradrenaline) on the oocytes of sea urchins. This article has absolutely no logical relationship to the stimulation in the growth rate of bacteria or viruses, and none was implied by the article. The reference could neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a rejection under 35 U.S.C. 103.

E. Sukmanskii et al. teaches that certain hormones decreased the mitotic index of certain L-cells (reported in the NCBI PubMed QUERY printout as mice cells). The decrease of mitotic activity in mouse L-cells has absolutely no logical relationship to the stimulation in the growth rate of bacteria or viruses, and none was implied by the article. The reference could neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a rejection under 35 U.S.C. 103.

It was absolutely clear to me at the time of reviewing the rejections under 35 USC 102/103 that the rejections were clearly in error with respect to the invention which I thought was being claimed at the time. With the clear instructions and explanations that I gave the attorney, I still do not understand why the rejection was not argued and readily overcome. It was only upon seeing the actual claims which issued in the LYTE Patent on May 13, 1997 that I became aware of and appreciated the error that there were no claims in the LYTE Patent which covered the important invention of enhancing the growth of bacteria and viruses and harvesting by-products of the bacteria and virus.

STATEMENT SATISFYING 37 C.F.R. §1.175(a)(6)

The errors specifically discussed herein arose without any deceptive intention on the part of the applicant. I offer to surrender U.S. Patent No. 5,629,349 to the Patent and Trademark Office in order to obtain a Reissue of that Patent.

STATEMENT SATISFYING 37 C.F.R. §1.175(a)(7)

I acknowledge that I have a duty to disclose information of which I am aware which is material to patentability and the examination of this reissue application in accordance with Title 37, Code of Federal Regulation, Section 1.56.

I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorney to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Mark A. Litman, Registration No. 26,390.

Send correspondence to:

Mark A. Litman
Schwegman, Lundberg, Woessner & Kluth, P.A.
1600 TCF Tower
121 South Eighth Street
Minneapolis, MN 55402

Direct telephone calls to:

Mark A. Litman
(612) 373-6975

Respectfully submitted,

Dated: 2/1/99

Mark Lyte

REISSUE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mark Lyte	
Serial No.:	Unknown	
Filed:	Concurrently Herewith	Docket: 933.001USR
Title:	COMPOUNDS FOR MODULATING GROWTH OF INFECTIOUS AGENTS	

ASSENT BY ASSIGNEE UNDER 37 C.F.R. § 1.172 AND POWER OF ATTORNEY

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

BioNutrix, LLC, being the Assignee of the entire interest in and to U.S. Patent No. 5,629,349, issued on May 13, 1997, to Mark Lyte and entitled "Compounds for Modulating Growth of Infectious Agents", hereby assents to the reissue of said patent.

Assignee hereby appoints:

Anglin, J. Michael	Reg. No. 24,916	Klima-Silberg, Catherine I.	Reg. No. 40,052
Arora, Suneel	Reg. No. 42,267	Kluth, Daniel J.	Reg. No. 32,146
Bianchi, Timothy E.	Reg. No. 39,610	Lacy, Rodney L.	Reg. No. 41,136
Billion, Richard E.	Reg. No. 32,836	Leffert, Thomas W.	Reg. No. 40,697
Black, David W.	Reg. No. 42,331	Lemaire, Charles A.	Reg. No. 36,198
Brennan, Thomas F.	Reg. No. 35,075	Litman, Mark A.	Reg. No. 26,390
Brooks, Edward J., III	Reg. No. 40,925	Lundberg, Steven W.	Reg. No. 30,568
Clark, Barbara J.	Reg. No. 38,107	Mates, Robert E.	Reg. No. 35,271
Drake, Eduardo E.	Reg. No. 40,594	McCrackin, Ann M.	Reg. No. 42,858
Dryja, Michael A.	Reg. No. 39,662	Padys, Danny J.	Reg. No. 35,635
Embretson, Janet E.	Reg. No. 39,665	Polglaze, Daniel J.	Reg. No. 39,801
Fogg, David N.	Reg. No. 35,138	Schwegman, Micheal L.	Reg. No. 25,816
Forrest, Bradley A.	Reg. No. 30,837	Sieffert, Kent J.	Reg. No. 41,312
Harris, Robert J.	Reg. No. 37,346	Slifer, Russell D.	Reg. No. 39,838
Holloway, Sheryl S.	Reg. No. 37,850	Terry, Kathleen R.	Reg. No. 31,884
Huebsch, Joseph C.	Reg. No. 42,673	Viksnins, Ann S.	Reg. No. 37,748
Kalis, Janal M.	Reg. No. 37,650	Woessner, Warren D.	Reg. No. 30,440

as its attorneys, with full power of substitution and revocation, to prosecute the reissue application, to make alterations and amendments therein, and to transact all business in the U.S.

REISSUE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mark Lyte	
Serial No.:	Unknown	
Filed:	Concurrently Herewith	Docket: 933.001USR
Title:	COMPOUNDS FOR MODULATING GROWTH OF INFECTIOUS AGENTS	

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

REISSUE APPLICATION
CLAIMS AMENDMENT AND DISCUSSION

In connection with the application for reissue of U.S. Patent No. 5,629,349, submitted concurrently herewith, the following are the proposed Reissue Claims to be added:

3. *A method of enhancing the growth of bacteria or viruses in a host medium said host medium being selected from the group consisting of in vitro and cell cultures, said method comprising the introduction of an effective amount of a catecholamine to the host medium to enhance the growth of said bacteria or viruses.*

4. *The method of claim 3 wherein the introduction of said catecholamine acts directly on enhancing the growth of said bacteria or virus.*

5. *The method of claim 3 wherein the growth of a bacteria is enhanced and said bacteria is a Gram-positive bacteria.*

6. *The method of claim 3 wherein the growth of a bacteria is enhanced and said bacteria is a Gram-negative bacteria.*

7. *The method of claim 3 wherein said catecholamine is selected from the group consisting of norepinephrine, epinephrine, and dopamine.*

8. *The method of claim 4 wherein said catecholamine is selected from the group consisting of norepinephrine, epinephrine, and dopamine.*

9. *The method of claim 5 wherein said catecholamine is selected from the group consisting of norepinephrine, dopamine and epinephrine.*

10. *A method of enhancing the growth of Gram-negative bacteria in a host medium said host medium being selected from the group consisting of in vitro and cell cultures, said method comprising the introduction of an effective amount of a catecholamine to the host medium to enhance the growth of said Gram-negative bacteria.*

11. *The method of claim 10 wherein said catecholamine is selected from the group consisting of norepinephrine, epinephrine and dopamine.*

12. *A method for harvesting the by-products of enhanced growth of bacteria or viruses comprising introducing an effective amount of a catecholamine to an in vitro or cell culture host medium of bacteria or virus to act directly on enhancing the growth of said bacteria or viruses, and collecting by-products generated by said bacteria or viruses.*

13. *The method of claim 12 wherein said introduction of said catecholamine acts directly on enhancing the growth of said bacteria or virus.*

14. *The method of claim 12 wherein a Gram-negative bacteria undergoes said enhanced growth.*

15. *The method of claim 13 wherein a Gram-negative bacteria undergoes said enhanced growth.*

16. *The method of claim 14 wherein said Gram-negative bacteria is selected from the group consisting of E. coli and Y. entercolitica.*

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17. *The method of claim 13 wherein an inhibitor is determined which intercedes at any point in a catecholamine biosynthetic pathway, and Gram-negative bacteria are subsequently treated by said inhibitor.*

18. *The method of claim 14 wherein an inhibitor is determined which intercedes at any point in a catecholamine biosynthetic pathway, and Gram-negative bacteria are subsequently treated by said inhibitor.*

19. *The method of claim 13 wherein said catecholamine is selected from the group consisting of norepinephrine, epinephrine, and dopamine.*

20. *The method of claim 14 wherein said catecholamine is selected from the group consisting of norepinephrine, epinephrine, and dopamine.*

21. *A method for harvesting the by-products of enhanced growth of bacteria or viruses comprising introducing an effective amount of a catecholamine to an in vitro or cell culture host medium of bacteria or virus to act directly on enhancing the growth of said bacteria or viruses, and collecting by-products other than glucose generated by said bacteria or viruses.*

22. *The method of claim 21 wherein said enhanced growth is effected on bacteria and said bacteria comprises Gram-negative bacteria.*

REMARKS CONCERNING THE PROSECUTION HISTORY OF THE APPLICATION WHICH ULTIMATELY ISSUED AS U.S. PATENT NO. 5,629,349

The original U.S. Patent Application (07/847196, filed March 6, 1992), the File Wrapper Continuation of which ultimately issued as U.S. Patent No. 5,629,349 (hereinafter the "LYTE Patent"), had a claim therein which was as follows:

"1. A method for affecting the growth of vectors and cell cultures and living organisms, said vectors and cell cultures and living organisms being characterized by the presence of at least

on catecholamine receptor site, said method comprising the steps of:

administering at least one chemical compound, said chemical compound selected from among the group of catecholamine receptor site agonists, catecholamine site receptor antagonists, catecholamine analogs, catecholamine derivatives, and mixtures thereof; and

administering said chemical compound in an amount sufficient to effect a desired level of growth of said vectors and living organisms."

These claims were rejected in an Office Action mailed on September 25, 1992 for the following grounds and reasons:

I. Claims 1-23 were rejected under 35 U.S.C. 102/103 as unpatentable over Dyer et al. Or Moger et al. It was asserted that each of the references teaches the affecting of the growth of a vector or cell culture using a catecholamine. This was asserted to be what the Applicant was claiming, and therefore the claims were asserted to not be patentable.

II. Claims 1-23 were rejected under 35 U.S.C. 112, second paragraph as failing to particularly point out and distinctly claim the invention. Certain terms such as "vectors," "analogs" and "derivatives" were held to be indeterminate.

III. Claims 1-23 were rejected under 35 U.S.C. 102/103 as unpatentable over Kotimchenko et al. Or Sumanskii et al. Each of the references was asserted to show the use of a neurotransmitter chemical to affect the growth of "living organisms." No patentable distinction was seen between the process of the references and the process of the claims.

The response to this Office Action (filed on December 21, 1992) filed new claims 24-33. A restriction requirement, and an asserted constructive election was erroneously made, the restriction was made as between:

I. Claims 29-33 drawn to a method of diagnosis and glucose production, these claims being held to have been constructively non-elected since they were held to have been not previously examined and their subject matter is new. This assertion was clearly in error, as these claims are within the scope of original claim 1 filed in the Application and reproduced above.

II. Claims 24-25 drawn to methods of suppressing growth.

III. Claims 26-28 methods of suppressing growth with a catecholamine blocker.

The attorney of record canceled claims 29-33 and elected claims 24 and 25 for prosecution on the merits. These claims were rejected, an Amendment after Final Rejection was filed and refused admission by an Advisory Action. The Application was then refiled as a File Wrapper Continuation, with only claims 24-28 present in the Application.

It is to be noted that the "constructive election" (and hence a purported 'constructive restriction requirement') were not established under any legal standard, and that there is no regulatory or case law basis which attorney for applicant has found to allow such a practice. The MPEP, Chisum on Patents, and a Lexis-Nexis search on Federal case law after 1944 (searching for restriction and construct! W/3 elect!) found no citations on the topic. There apears to have been no basis for the "constructive election" and any attempt at a restriction requirement along the lines done would have been error and not a "proper restriction" requirement.

A true generic claim 1 as filed in the Application had been present, all of the claims subsequently submitted were subgeneric to that claim, and so the broad subject matter had been examined contrary to the position asserted in the Office Action to justify the "constructive election" which finds no basis in regulation, rule, statute or case law in the manner presented and with the facts presented. There was, therefore, no proper restriction requirement and no election of any sort against the subject matter presently encompassed by the claims for Reissue Patent filed with this application.

A potentially proper restriction requirement was then filed between claims 24-25 and 26-28 in an Office Action mailed January 31, 1995. These two groups of claims were directed at slightly different variants of suppression methods operating on Gram-positive bacteria, and neither of these methods are included within the scope of the new claims submitted for examination in this Reissue Application. Applicant, through his attorney of record, then elected claims 24-25 for prosecution on the merits. These claims were then rejected under 35 U.S.C.

112, first and second paragraphs. After another series of rejections, with only claims 24 and 34 in the Application, the two claims in the LYTE Patent were issued.

As is evident from the above comparison, original claim 1 of the LYTE Patent is limited to the method when employed to inhibit the growth of Gram-positive bacteria *in vitro* or in a cell culture. That is an extremely narrow process of little commercial utility. The original application as filed on March 6, 1992 clearly identified the scope of the invention as including enhancing the growth of bacteria and viruses *in vitro* and in cell cultures. Therefore, the LYTE patent is believed to be defective. New claim 3 recites no such limitations with respect to inhibiting growth in only Gram-positive bacteria. Accordingly, independent claim 3 is submitted to properly claim the broadest improvement over the methods of the prior art to which the patentee is entitled.

It is important to note that no restriction requirement in the Application filed on June 27, 1994 was ever asserted against the claims presented in the Reissue Application, so there is no applicability of issues found in *In re Orita, Yahagi, and Enomoti*, 193 USPQ 145, where it was held that "Although appellants undoubtedly erred by failing to file a timely divisional application in order to obtain a divisional patent, it does not follow that such error caused the original patent to be 'partially inoperative by reason of the patentee claiming less than he had a right to claim in the patent' as appellants aver in their reissue declaration under 37 CFR 1.175..." It was further stated in *In re Orita* that "...granting reissue claims substantially identical to those non-elected in application I would be ignoring the proper restriction requirement set forth in that application in which appellants acquiesced. Indeed, appellants' misapplication of section 251 would, if permitted, circumvent the copendency requirement of section 120 incorporated by reference in section."

The original restriction requirement was against

1) a method of diagnosing the presence of Gram-negative bacteria, including specific physical steps, none of which are recited in the claims of the Reissue Application;

2) a method of producing glucose from a lactose broth, the claim reciting specific physical steps which are not recited in the claims of the Reissue Application;

3) a method for suppressing the growth of Gram-positive bacteria; and

4) a specific method for suppressing the growth of Gram-negative bacteria comprising the introduction of an effective blocker of catecholamine receptor sites of the organisms.

Methods 1), 2), 3) and 4) are clearly outside the scope of the claimed subject matter of the Reissue Application.

In comparing new claim 3 to original claim 1, claim 1 recites the step of "suppressing the growth of Gram-positive bacteria." Claim 3, however, does not include such a limitation, but instead recites "...enhancing the growth of bacteria or viruses.." This claim is not limited to suppression of growth, but only to enhancing of growth. The only actual restriction requirement which occurred in the prosecution of the U.S. Patent Application U.S. Serial No. 08/266,805 filed on June 27, 1994 was between:

I. Claims 24 and 25, drawn to a method of suppressing the growth of Gram-positive organisms with an amount of catecholamine, classified in Class 514, subclass 727.

II. Claims 26-28, drawn to a method of suppressing the growth of Gram-negative organisms by the introduction of an effective blocker of catecholamine receptor sites of the organisms, classified in Class 514, subclass 224.8.

The constructive election against claims 29-33 found in the parent application preceding U.S. Patent Application U.S. Serial No. 08/266,805 was not a proper restriction requirement, and was substantively incorrect even in its substance. In any event, the claims of the Reissue Application were not the subject of restriction requirements in U.S. Patent Application U.S. Serial No. 08/266,805. Therefore the claims submitted in this Reissue Application do not read on any species, elected invention or non-elected invention for which a proper restriction requirement was made.

In a similar comparison, independent method claim 12 was compared in the Reissue Declaration to original method claim 1 for purposes of discussion.

As is evident from that comparison, original claim 1 of U.S. Patent No. 5,629,349 is limited to the method when employed to inhibit the growth of Gram-positive bacteria. Accordingly, the claims exclude any useful method of increasing the supply of by-products from said bacteria or cell. The new claim 12 submitted in the Reissue Application encompasses this

useful method associated with the enhancement of bacteria or virus growth.

Each of reissue claims 4 through 11 and 13-20 depend from either reissue claim 3 or reissue claim 12, respectively. None of reissue claims 4-11 or 13-20 recite a limitation that reads on the subject matter which was restricted, non-elected, and effectively abandoned in the prosecution of U.S. Patent Application U.S. Serial No. 08/266,805 which ultimately issued as U.S. Patent No. 5,629,349 (hereinafter, the "Original Application"). Accordingly, reissue claims 4-11 and 13-20 differ from original claims 1 and 2 and are the proper subject matter for a Reissue Application.

The rejections in the Office Action in U.S. Patent Application U.S. Serial No. 08/266,805 mailed on September 25, 1992 included rejections of all claims (Claims 1-23) under 35 U.S.C. 102/103 as being unpatentable over Dyer et al., Moger et al., Kotimchenko, or Sukmanskii et al. These rejections were clearly explained to the attorney of record as being completely erroneous, at least for the following reasons:

A. The invention intended to be claimed was the enhanced growth of bacteria or viruses by the administration of catecholamines *in vitro* or in cell cultures. The enhanced growth was a result of the addition of the catecholamines.

B. Dyer et al. showed the stimulation of androgen production in ovarian cells when cultured in a serum-free medium. The mechanism proposed in the Chem Abstracts article was that the "catecholamine-augmented androgen prodn. Provides a direct link between the autonomic nervous system and regulation of ovarian steroid synthesis." That explanation has no logical bearing or relationship to the stimulation in the growth rate of bacteria or viruses, and none was implied by the article. The reference can neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a rejection under 35 U.S.C. 103.

C. Moger et al. teach that catecholamines stimulated androgen production by mouse interstitial cells in primary culture. The Chem Abstract text has no suggestion on the effect of catecholamine with respect to any cell growth, but only on the stimulation of androgen production. Again, that article has no logical bearing or relationship to the stimulation in the growth rate of bacteria or viruses, and none was implied by the article. The reference can neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a

rejection under 35 U.S.C. 103.

D. Khotimchenko describes the effect of adrenotropic substances (including ephedrine and noradrenaline) on the oocytes of sea urchins. This article has absolutely no logical relationship to the stimulation in the growth rate of bacteria or viruses, and none was implied by the article. The reference can neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a rejection under 35 U.S.C. 103.

E. Sukmanskii et al. teaches that certain hormones decreased the mitotic index of certain L-cells (reported in the NCBI PubMed QUERY printout as mice cells). The decrease of mitotic activity in mouse L-cells has absolutely no logical relationship to the stimulation in the growth rate of bacteria or viruses, and none was implied by the article. The reference can neither anticipate the invention of Reissue Claims 3 and 12, nor provide a *prima facie* basis for a rejection under 35 U.S.C. 103.

As can be seen from this analysis, the rejections of record were completely in error and should have been directly responded to on a substantive basis. In any event, the present claims of the Reissue Application contain narrowing limitations which effectively and clearly overcome those rejections.

The Examiner is invited to telephone the below-signed attorney at 612-373-6975 to discuss any questions which may remain with respect to the present application.

Respectfully submitted,

MARK LYTE

By his Representatives,

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Date 1 February 1999 By ______________________
Mark A. Litman
Reg. No. 26,390

CERTIFICATE UNDER 37 CFR 1.10:
"Express Mail" mailing label number: EM287852441US Date of Deposit: February 1, 1999
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Chris Hammond
Name

Chris Hammond
Signature

REISSUE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mark Lyte		
Serial No.:	Unknown		
Filed:	Concurrently Herewith	Docket:	933.001USR
Title:	COMPOUNDS FOR MODULATING GROWTH OF INFECTIOUS AGENTS		

COMMUNICATION RE: TRANSMITTAL OF REISSUE APPLICATION

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please find enclosed herewith an application for reissue of U.S. Patent No. 5,629,349, issued on May 13, 1997, to Mark Lyte and entitled "Compounds for Modulating Growth of Infectious Agents", which reissue application includes the following:

1. Reissue Application including Specification, Claims, Abstract, and Drawings;
2. Declaration [of Inventor] for Broadening Reissue Application;
3. Offer to Surrender Original Patent;
4. Assent by Assignee and Power of Attorney;
5. Request to Transfer Drawings and copy of drawings as printed in patent of parent application;
6. Information Disclosure Statement, Form 1449, and copies of cited references;
7. Claims Amendment and Discussion;
8. Reassignment Agreement, recordation cover sheet, and check to pay recordation fee;
9. Contribution Agreement, recordation cover sheet, and check to pay recordation fee; and
10. A check to pay the reissue filing fee.

Please charge any additional required fees, or credit overpayment, to Deposit Account No. 19-0743.

Respectfully submitted,

MARK LYTE

By his Representatives,

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Date 1 February 1999 By [signature]
Mark A. Litman
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CERTIFICATE UNDER 37 CFR 1.10:
"Express Mail" mailing label number: EM287852441US Date of Deposit: February 1, 1999
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Chris Hammond
Name

Chris Hammond
Signature

COMPOUNDS FOR MODULATING GROWTH OF INFECTIOUS AGENTS

The findings reported herein were funded by the National Institute of Health, under grant MH-45246, and accordingly the United States Government has acquired ownership of certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation of application Ser. No. 07/847,196, filed Mar. 6, 1992 (now abandoned), which is itself a continuation-in-part of application Ser. No. 07/753,709, filed Sep. 3, 1991 (now abandoned), which is itself a continuation-in-part of application Ser. No. 07/730,485, filed Jul. 16, 1991 (now abandoned).

BACKGROUND OF THE INVENTION

I. Field of the Invention

This invention relates generally to a method of modulating the proliferation of microorganisms or other infectious vectors and, more particularly, to a method of introducing a neurochemical to augment, repress or otherwise affect the growth of Gram-reactive organisms. The process involves the introduction (or application) of effective amounts of a group of neurochemicals known as the catecholamines. Each microorganism tested has been shown to have specific requirements for one or more of the subject catecholamines. Depending on the catecholamine being employed, suppression or enhancement of growth may be effected. Furthermore, growth regulation can be effected in vivo and/or in vitro. The characterization of the receptor through which the catecholamine binds to the cell or cellular components as novel, enables further control of cell growth by the application of either receptor agonists or antagonists specific for this novel receptor.

II. Discussion of the Prior Art

Sepsis is the generally febrile pathologic state resulting from the presence of microorganisms or their poisonous products in the blood stream particularly in humans or other mammals. It occurs when spreading infectious agents are not successfully arrested within the lymph nodes, and thus directly invade venous channels. Although it is not uncommon to have periodic invasions of bacteria into the blood stream, called bacteremia, these outbursts are normally handled quickly and effectively by macrophages circulating in the blood. However, in some circumstances, such large numbers of bacteria may be involved in the invasion that the macrophages are overwhelmed and under-effective, resulting in the symptoms of fever, chills, general malaise and lethargy known as septicemia. In aggravated cases, it is possible that organisms reach such a high population within the circulation that they circulate in clumps. Under these conditions, it is possible that these circulating clumps may lodge within organs and produce large numbers of microabscesses, a situation which is called septicoyemia.

Traditional treatment of sepsis, septicemia, or septicoyemia is performed using antimicrobial agents, typically in conjunction with the use of vasoactive drugs such as norepinephrine and dopamine. These antimicrobial agents are typically designed to affect bacterial wall structure or bacterial metabolic processes. Such agents may be particularly targeted at a specific metabolic process or cellular receptor. However, despite this specificity, conventional antimicrobial therapy is unsuccessful in a large number of cases. In particular, it has been documented that up to 60%

of all patients diagnosed with sepsis eventually succumb to the condition. Thus, despite the great strides forward, there remains great need for more specific and more effective antimicrobial agents or treatment protocols.

The primary role for the use of vasoactive drugs in septic conditions has been the restoration of normal hemodynamics. A typical clinical picture involving sepsis is the patient who fails to respond to traditional treatment or who undergoes intensive antibiotic therapy and apparent resolution of sepsis, with improvement in condition two to three days post admission to hospital. However, nearly 40% of patients relapse on the third to fourth day of treatment, with death ensuing rapidly thereafter. My recent data indicates that the use of catecholamines (such as norepinephrine and dopamine) as the preferred vasoactive agents may, in fact, be an essential factor contributing to the worsening clinical condition. My data indicates that administration of catecholamines to patients in these conditions results in providing some bacteria with a potent growth stimulus that permits rapid proliferation bacteria and eventual death of the patient, even in the face of appropriate antibiotic therapy.

Catecholamines in humans are a class of hormones that evoke a response by activation of adenyl cyclase. These compounds are targeted to specific hormone receptors in mammals and produce varied responses, depending upon the nature of the target tissue. The majority of catecholamines evoke their characteristic response by influencing the activity of pre-existing cellular enzyme systems. Thus, the response evoked may be almost instantaneous, such as in the case of neurotransmitters including norepinephrine and epinephrine. Norepinephrine, epinephrine and dopamine are the characteristic hormones of the mammalian sympathetic nervous system. All are amine derivatives of the catechol nucleus (dihydroxybenzene). These compounds have clearly identified peripheral effects. Classic feedback inhibition processes control the production of these compounds, in which the rate limiting step in the pathway is hydroxylation of the amino acid precursor tyrosine to form dihydroxyphenylalanine (dopa). In mammals, synthesis of catecholamines is a unique feature of sympathetic nervous tissue. However, in certain disease states, hormone producing tumors may release catecholamines directly into the circulation and, thus, manifest peripheral effects of these compounds as a result of plasma concentration instead of local tissue concentration. Other states, such as stress, are also classic activators of the production of catecholamines concomitant with the presumed suppression of the immune system. Otherwise, most manifest a non-circulatory effect.

Jones, et al., among others, have noted a severalfold increase in plasma norepinephrine and epinephrine levels during bacteremia. This increase has been attributed to activation of the adrenal medulla and peripheral sympathetic fibers. They suggest that plasma catecholamines may be an indicator of lethality, since in all reported instances, the mean values for plasma norepinephrine and epinephrine were higher in nonsurviving rats. The purpose of their study was to quantitate the levels of peripheral sympathetic activation as indicated by plasma catecholamine levels during sepsis. The actual reason for the increase was not the primary focus and possible blockage of re-uptake at neuronal sites was not addressed.

Although bacteria lack a nervous system and, thus, have no apparent need for neurotransmitters such as norepinephrine, epinephrine and dopamine, I have recently discovered that the presence of these chemicals in their environment may positively or negatively influence the growth of Gram-negative or Gram-positive bacteria. Based

upon this discovery, I have devised a method of treatment of the pathologic state of a patient in order to modulate the growth of such infectious agents, as well as regulate viral, phage, plasmid, microorganism, or parasite reproduction.

Such a class-dependent response presents great opportunity for novel approaches in drug design. For example, the identification of the receptors by which bacteria may use these neurochemicals leads to the design of receptor antagonists which may be as potent in the control of bacterial growth as current antimicrobial therapy, including the application of antibiotics. Gram-negative bacteria having a growth-enhancing response to neurochemicals are open to treatment by any inhibitor which intercedes at any point in the catecholamine biosynthetic pathway, such as monoamine oxidase inhibitors, in order to interrupt specific steps in the conversion pathways of these catecholamines.

It is accordingly a principal object of the present invention to provide a new and improved method for the treatment of sepsis, septicemia or septicoyemia.

Another object of the present invention is to provide a new and improved method for the treatment of living patients suffering the effects of a microbial, parasitic or viral infection.

It is yet another object of the present invention to provide a new and improved method for enhancing or suppressing the proliferation of microbial or viral agents or vectors in a living system.

A further object of the present invention is to provide a new and improved method for the suppression of bacterial replication within living patients.

Another object of the present invention is to provide a new method for specifically binding the novel receptor identified herein.

Yet another object of the present invention is to provide a new method for application in the field of the design of drugs and therapeutics, by which recognition of this novel receptor identified herein is useful in suppressing the proliferation of infectious agents.

SUMMARY OF THE INVENTION

The foregoing objects and advantages of the invention are achieved by providing a method of applying a neurochemical to a substrate or living patient for the purpose of modulating the growth of infectious agents such as bacteria, parasites or viruses. I have found that the addition of such neurochemicals to cultures of Gram-negative bacteria, such as *Escherichia coli* and *Yersinia enterocolitica*, can cause vast increases in bacterial growth capacity, depending upon the concentration of neurochemical and initial bacterial load in the culture. The reaction involved is very specific, since a similar response has not been evoked by any of the metabolites of norepinephrine or epinephrine, except in *E. coli*. Conversely, these catecholamines can suppress the growth of Gram-positive bacteria, such as *Staphylococcus aureus*. This class-dependent response heretofore unrecognized presents great opportunity for novel approaches in drug design.

The modulation of bacterial growth is effected in vitro by the addition of one or more catecholamines to buffered basal culture medium containing the subject bacterial cells and control or catecholamine solution in an amount ranging from about 10^{-4} to 10^{-10}M concentration. Modulation of growth rate can be detected by a number of standard methodologies. For example, scintillation counts of ^{3}H-thymidine, optical density readings or plate counts may be performed. In order

to examine whether the ability of the catecholamines is modulated by known receptors, known antagonists which specifically block receptor binding of catecholamines, such as norepinephrine and epinephrine, may be added to bacterial cultures in the presence or absence of a preselected concentration of norepinephrine. Known agonists are also tested.

Accordingly, a method of treatment of infections has been devised in which treatment protocol is based not upon whether the causative agent is infectious, but upon the nature of the cell wall in microorganisms (or other binding characteristics in viruses or vectors). It has been discovered that growth is enhanced in Gram-negative microbes when in the presence of certain catecholamines. Thus, the accepted practice of administering catecholamines in such patients should be suspended. However, growth of Gram-positive bacteria is suppressed in these conditions, which contraindicates suspension. Because the affinity of these microorganisms for certain catecholamines is receptor-mediated, as demonstrated herein, the method of treatment of infections caused by these organisms is directed at manipulation of the receptor. Furthermore, the receptor involved is not blocked by known α_1, α_2 or β adrenergic agents.

These discoveries have important industrial applications as well. Thus, a method of including one or more catecholamines in the culture medium for such organisms is described. When growth is stimulated, the production of desirable by-products is augmented. The method of stimulating this growth is directed at the specific receptor involved.

Other objects of the present invention and many of its attendant advantages will be more readily appreciated as the invention becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings in which like reference numerals designate like parts throughout.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a block diagram of a preferred embodiment of the method of the present invention for in vitro applications;

FIG. 2 is a block diagram of an alternative embodiment of the present invention for in vivo applications;

FIG. 3 is a plot illustrating differing rates of thymidine incorporation into newly synthesized DNA when *E. coli* is cultured in various concentrations of norepinephrine, epinephrine and dopamine at an initial inoculum of 15 CFU per well;

FIG. 4 is a plot similar to FIG. 3 illustrating differing rates of ^{3}H-thymidine incorporation into newly synthesized DNA when *E. coli* is cultured in various concentrations of norepinephrine, epinephrine and dopamine at an initial inoculum of 1500 CFU per well;

FIG. 5 is a plot illustrating the differing rates of ^{3}H-thymidine incorporation into newly synthesized DNA when *Y. enterocolitica* is cultured in various concentrations of norepinephrine, epinephrine and dopamine at an initial inoculum of 10,000 CFU per well;

FIG. 6 is a graph illustrating the change in optical density with various concentrations of norepinephrine in a culture of *E. coli* with an initial inoculum of 25 CFU per well;

FIG. 7 is a graph illustrating pour plate counts for cultures of *P. aeruginosa* cultured in various concentrations of norepinephrine;

FIG. 8 is a plot illustrating differing rates of thymidine incorporation into newly synthesized DNA when *S. aureus*

is cultured in various concentrations of norepinephrine, epinephrine and dopamine at an initial inoculum of 1600 CFU per well;

FIG. 9 is a plot similar to FIG. 8 illustrating the suppression of growth of *E. coli* in the presence of the anti-adrenergic compound chlorpromazine with an initial inoculum of 2200 CFU per well;

FIG. 10 is a plot similar to FIGS. 8 and 9 illustrating the lack of an effect of adrenergic receptor agonists octopamine and ephedrine on the growth of *E. coli* with an initial inoculum of 25 CFU per well;

FIG. 11 is a plot similar to FIG. 10 illustrating the growth enhancing effect of high concentrations of the β-receptor agonist isoproterenol on the growth of *E. coli* at an initial inoculum of 25 CFU per well;

FIG. 12 is a plot illustrating the lack of an effect of the α-adrenergic receptor antagonist benextramine tetrachloride on growth of *E. coli* with an initial inoculum of 30 CFU per well;

FIG. 13 is a plot illustrating the lack of a growth enhancing effect of high concentrations of the β-adrenergic receptor antagonist alprenolol on the growth of *E. coli* at an initial inoculum of 25 CFU per well;

FIG. 14 is a plot similar to FIG. 10 illustrating a lack of decrease in growth of *S. aureus* in the presence of the dual α- and β-adrenergic receptor agonist ephedrine at an initial inoculum of 27 CFU per well;

FIG. 15 is a graph illustrating the increase in glucose production at increasing concentrations of norepinephrine in cultures of *E. coli;*

FIG. 16 is a plot illustrating the increase in β-galactosidase activity with increasing concentrations of norepinephrine in cultures of *E. coli;*

FIG. 17 is a graph illustrating a ^{3}H-norepinephrine competition/displacement curve;

FIG. 18 is a saturation curve for ^{3}H-norepinephrine;

FIG. 19A is a competition/displacement curve illustrating the non-displacement with non-specific binding which occurs with the addition of ^{3}H-prazosin;

FIG. 19B is a competition/displacement curve illustrating the non-displacement with non-specific binding which occurs with the addition of ^{3}H-rauwolscine;

FIG. 19C is a competition/displacement curve illustrating the non-displacement with non-specific binding which occurs with the addition of ^{3}H-dihydroalprenolol;

FIG. 20 is a tissue dependency graph demonstrating saturation in which all of the novel receptors are saturated with catecholamines at high concentrations of bacteria;

FIG. 21 contains Table 1 which depicts data representative of the contents of FIGS. 3–5 and further includes data on the effective administration of catecholamine metabolites and is also referred to as "Table 1"; and

FIG. 22 contains Table 2 which demonstrates the effective norepinephrine and various α- and β-receptor agonists on the growth of *Y. enterocolitica* and is also referred to as "Table 2".

DESCRIPTION OF THE PREFERRED EMBODIMENT

Sepsis, or any of the related septic injuries or conditions, evokes a multiplicity of organ system responses, some of which are directed at maintaining cardiovascular and metabolic homeostasis. Concomitant, increased peripheral sympathetic nerve activity may result in an increase in circulat-

ing catecholamines in order to stimulate peripheral adrenergic receptors to support the cardiovascular and metabolic processes which may be interrupted or impaired by sepsis. Thus, it is common to administer norepinephrine and dopamine during a period of sepsis in order to enhance and stabilize hemodynamics. Cardiovascular processes may be enhanced by augmented cardiac output, whereas other metabolic processes may be improved by, for example, increased mobilization of liver glycogen.

Observation of the typical post-hospitalization outcome of such patients indicates that following an initially successful response to antimicrobial therapy, there is a re-infection effect that eventually results in the death of up to 60% of this population. Previously, these deaths have been attributed to nonspecific causes such as generalized organ failure. It has been noted, however, that a vast majority of these deaths have followed a Gram-negative infection and that the primary causative vector was *E. coli*. The present invention is concerned with a method of treatment of these patients that for the first time explains this rebound effect in terms of environmental conditions previously believed to be nonsupportive of microbial growth, but which, in fact, actually augment growth, as described hereinafter.

The modulation of microbial growth may be effected in vitro by the addition of one or more catecholamines in an amount ranging from about 10^{-4} to 10^{-10}M concentration to culture medium containing the subject microbial cells. Depicted at Block **1** of FIG. **1**, the culture medium should preferably be a relatively simple medium so as to facilitate the inclusion of the catecholamines in a known concentration. For example, a basal medium consisting of various constituents such as dextrose (0.5 g/l, 2.78 mM), ammonium nitrate (0.5 g/l, 6.25 mM), potassium phosphate (0.25 g/l, 1.84 mM), potassium chloride (0.25 g/l, 3.35 mM) and magnesium sulfate (0.25 g/l, 1.01 mM) adjusted to a final pH of 7.5. Additionally, hepes buffer may be added at a final concentration of 10 mM to provide additional buffering capacity. This medium may then be further supplemented with 30% V/V of bovine serum, although equivalent results may be obtained with serum from other sources such as pig, mouse and man. Suitable antioxidants such as ascorbic acid may also be added to prevent oxidation of catecholamines. However, such compounds should be used judiciously, since the antibacterial effects of antioxidants are well recognized. It is recognized that individual cells may require different basal medium preparations to insure optimum growth in catecholamine supplemented medium.

As depicted at Block **2** of FIG. **1**, the desired catecholamine is then dissolved in an appropriate amount of chilled boiled water. The catecholamine solution is then added to the basal medium in a final volume-to-volume addition of, for example, 20%. The subject microbial cells are prepared in serum supplemented basal medium at varying concentrations of 15 to 15,000 CFU per ml and as depicted at Block **3**, 100 μl of suspensions may be added into the wells of standard tissue culture plates. Next, 100 μl of catecholamine or control solution is added to the wells containing, for example, bacteria and the plates are incubated for varying lengths of time at an appropriate temperature in a humidified incubator, as at Block **4**. This process may be altered as necessary for application to a variety of non-microbial vectors as well.

Modulation of growth rate can be detected by a number of standard methodologies. For example, the cultures can be further supplemented at time of culture initiation by the addition of 50 μl of a 20 μCi solution of ^{3}H-thymidine in PBS per 200 μl of culture. Culture contents can then be

harvested onto filter mats using standard plate harvesters which separate unbound radioactive material from radioactive material that has been incorporated into the cell's DNA. The filter mats with bound material are then counted in a scintillation counter. The raw counts resulting from incorporation of the ^{3}H-thymidine into newly synthesized DNA is used as measure of cell proliferation. The greater the counts, as compared to cells incubated in the absence of catecholamines, the larger the enhancement of proliferation, and vice versa. Alternatively, contents of plate wells may be gently resuspended and the optical density readings taken in a standard microplate reader at an optical density of 550 nm or 630 nm. Increases in optical density readings in catecholamine supplemented wells as compared to control wells indicates greater mass of cells due to increased proliferation. Further, plate counts may also be performed by standard pour-plate methodology in which the contents of the plate well are gently resuspended and an aliquot removed and serially diluted samples are plated. After 24–48 hours, the number of colonies are enumerated with an increase in colonies relative to control plates indicating enhanced cell proliferation.

As depicted at Block **5**, certain by-products may arise as a consequence of the growth of microbes in appropriately selected media. For example, the growth of *E. coli* in a lactose broth will yield glucose. In the stimulating presence of catecholamines, as documented more fully hereinafter, this yield is increased due to an increase in proliferation. Thus, increases in such yield have great commercial potential. In appropriate circumstances, the microbes themselves may be harvested, or their products may be used as intermediates for other products. An example of the later is the use of microorganisms containing a genome for the conversion of glucose to ethanol.

FIG. **2** depicts a method for in vivo treatment of infection using a suggested method of the present invention. As denoted at Block **1**, the causative nature of the infection must first be assessed. Once the microbe, virus or vector has been identified, the appropriate therapy is commenced, as at Block **2**. This therapy would include either application of antimicrobial or antiparasitic agents, or pertinent anti-viral treatment. Depending upon the nature of the causative organism and resultant effects or hemodynamics, a decision must be made as to whether or not to introduce systemic catecholamines to the patient, as designated at Block **3**. If the decision is reached that catecholamines should be applied, metabolic and cardiac functions should be monitored as denoted at Block **4**. As described further hereinafter, it is also appropriate at this time to screen for any increase in the concentration of microbes, whether Gram-negative or Gram-positive. The therapy commenced at Block **2** should be continued (Block **5**) with intermittent assessment and monitoring, as denoted at Block **4**. Otherwise, therapy should be discontinued (Block **6**). If, however, the decision is made that due to the nature of the causative organism, catecholamines should not be applied, subsequent steps denoted at Blocks **7** and **8** include monitoring of metabolic and cardiac functions as well as a continuation of appropriate anti-microbial or anti-viral therapy.

Concomitant to the steps described in Blocks **1** through **8**, appropriate steps are taken at Block **9** to identify the agent causing the infection. It is to be emphasized that these steps (Block **1** and Block **9**) are undertaken simultaneously, due to the customary time delay in arriving at a proper identification and the deleterious impact which would ensue if therapy were delayed. Once the agent is identified, it may be necessary to alter or adjust therapy accordingly, as at Block **10**.

The incorporation of circulating catecholamines, specifically norepinephrine and epinephrine, into mammalian neuronal fibers during re-uptake is accomplished via receptors. These include the α- and β-receptors and their constituent subtypes. In order to examine whether the ability of the catecholamines is modulated by known receptors, known antagonists which specifically block receptor binding of catecholamines, i.e., norepinephrine and epinephrine, are added to bacterial cultures in the presence or absence of a preselected concentration of norepinephrine. For example, the β-adrenergic receptor antagonist alprenolol hydrochloride (HCl) was dissolved in chilled boiled water in an amount ranging from 5×10^{-4} to 5×10^{-12}M. Norepinephrine was also dissolved in chilled boiled water in an amount of 5×10^{-5}M. To serum supplemented basal medium, alprenolol HCl and norepinephrine were added alone or in combination. The subject bacterial cells were prepared in serum supplemented basal medium at varying concentrations of 15 to 15,000 CFU per ml and 100 μl of suspensions were added into 96 well tissue culture plates. Next, 100 μl of the alprenolol HCl alone, norepinephrine alone, or combination of varying amounts of alprenolol HCl with a constant amount of norepinephrine was added to wells containing bacteria. Additionally, 50 μl of a 20 μCi ^{3}H-thymidine per ml of phosphate buffered saline was added to all wells. At preselected time intervals, optical density readings were taken and immediately thereafter, wells were harvested using a standard plate harvester. The amount of radioactive incorporation of the ^{3}H-thymidine was determined using a scintillation counter. The ability of the antagonist alprenolol HCl to block the norepinephrine induced enhancement of proliferation through blockage of the known β-adrenergic receptor would be reflected in a reduction in counts as compared to cultures supplemented with norepinephrine alone.

All data points in the following Figures represent mean quadruplicate cultures and all points have a standard error of the mean of less than or equal to 10%. Experimental methods include standard radioligand techniques to assess the incorporation of ^{3}H-thymidine into newly synthesized DNA. Control values are designated CON. Commercially available norepinephrine and epinephrine were obtained in the hydrochloride and bitartrate forms. Efficacy was not found to be form-dependent. The hydrochloride form of dopamine was used.

FIG. 3 depicts a positive correlation between increase in concentration of a Gram-negative bacterium and increased ^{3}H-thymidine incorporation (DPM) into newly synthesized DNA in the presence of the catecholamines norepinephrine, epinephrine and dopamine. Data presented are for a 10-hour culture of *E. coli* with an initial inoculum of 15 colony-forming units (CFU) per well. Of these catecholamines, it can be seen that norepinephrine is the most potent enhancer of growth at low initial concentration of Gram-negative bacterium.

To show that the effect depicted in FIG. 3 was not dependent upon the initial concentration of bacterium, experiments were repeated at a variety of initial concentrations. For the Gram-negative bacterium, *E. coli*, all concentrations revealed the same growth enhancing trend. Accordingly, FIG. 4 depicts a 10-hour culture of *E. coli* with an initial inoculum of 1500 CFU per well. When normalized for initial concentration, it becomes apparent that the percent increase in ^{3}H-thymidine incorporation (DPM) for each catecholamine is less than the percent increase depicted in FIG. 3 for a lower initial concentration. This is explained on the basis of normal growth dynamics in which the stationary

phase is approached in a shorter time when there is a heavy initial inoculum than when the initial inoculum is very light. Accordingly, any effect caused by a growth enhancing factor will appear greater in a light inoculum than in a heavy one.

A variety of Gram-negative organisms were tested under these conditions, and it was discovered that the growth enhancing effect of norepinephrine was consistent and not species-specific. For example, FIG. 5 depicts a 36 hour culture of *Yersinia enterocolitica* with an initial inoculum of 10,000 CFU per well. The data represented herein were selected because they presented typical values for incorporation of ^{3}H-thymidine into newly synthesized DNA at high concentration of catecholamine. The enhancing effect of catecholamines is representative of all tested Gram-negative bacteria at high initial concentrations. No Gram-negative organism was found which did not demonstrate a selective preference for one or more of the catecholamines tested.

Table 1 depicts data representative of the contents of FIGS. 3 through 5 and further includes data on the effect of administration of catecholamine metabolites. These data confirm the impression presented in FIGS. 3 through 5 that *Y. enterocolitica* is considerably more selective in substrate usage than *E. coli*. The ubiquitous nature of *E. coli* is borne out by the fact that it is even capable of fueling growth by utilization of the metabolites 4-hydroxy-3-methoxyphenylglycol piperazine salt (MHPG) and normetanephrine (NOR). Data represent mean DPM values with initial concentrations of 15 and 1500 CFU for *E. coli* and 80 CFU for *Y. enterocolitica*. The mean DPM values were obtained from quadruplicate cultures and the standard error was 10% or lower.

The growth-enhancing effect of norepinephrine on Gram-negative bacteria is not a method-dependent artifact. FIG. 6 depicts spectrometric data for a 20 hour culture of *E. coli* with an initial inoculum of 25 CFU per well measured at 630 nanometers. A separate blanking plate containing all media and chemicals, but lacking a bacterial inoculum, was measured to provide a correction factor for all concentrations of norepinephrine and control. The same enhancement of growth of *E. coli* was detected with this method as was shown using scintillgraphic data.

FIG. 7 further illustrates that the growth-enhancing capacity of norepinephrine in Gram-negative bacteria is not method dependent. In standard plate counts for a 20 hour culture of *Pseudomonas aeruginosa*, increasing concentrations of norepinephrine correlated to an increased number of mean CFU for increasing concentrations of norepinephrine as compared to a control medium lacking norepinephrine.

In dramatic contrast to the growth enhancing effect of catecholamines on Gram-negative bacteria, FIG. 8 demonstrates that these compounds have essentially the reverse effect in Gram-positive bacteria. This trend was consistent for all Gram-positive bacteria tested and exemplified by the plot in FIG. 8. A 20 hour culture of *Staphyloccus aureus* representing an initial inoculum of 1600 CFU per well manifested a decline in ^{3}H-thymidine uptake into new DNA. As concentration of catecholamines increased, suppression of growth increased.

In summary, it was noted that all Gram-negative organisms tested in these studies responded positively to the introduction of norepinephrine to their growth media. Enhancement of proliferation varied between organisms, with proliferation being the most pronounced for *E. coli*. This organism was sensitive to all catecholamines tested, in contrast to the majority of Gram-negative bacteria, which were sensitive only to the presence of norepinephrine and to

a lesser effect, dopamine. This effect was not method specific and was manifested at a slower rate as initial bacterial inoculum increased. In contrast, all Gram-positive bacteria tested showed a suppression of growth in the presence of the catecholamines tested. This effect was not dependent upon the initial inoculum concentration, nor on the duration of culture. Data were selected for presentation which demonstrate that the growth-enhancing effects were consistent throughout a range of conditions, and not limited to a narrow optimal range, although it is evident that certain bacteria have a selective preference for growth in the presence of one or more of the catecholamines normally available during septic crises. Furthermore, my preliminary data indicates that enhancement is not limited to bacteria and may be expected in viruses and other vectors as well.

In light of these findings, it was important to determine the effect of anti-adrenergic compounds on the growth of both Gram-negative and Gram-positive bacteria under similar conditions.

RECEPTOR

The underlying mechanism of this growth enhancement or suppression is important for the design of drugs to control sepsis. It is also important in regulation of the operons which control carbon utilization. The change in pathogenicity documented in FIGS. **3** through **8** involves receptor-mediated recognition of these catecholamines and leads to opportunity for the design of novel drugs to control growth, as described more fully hereinafter.

FIG. **9** depicts the effect of the anti-adrenergic compound chlorpromazine on the synthesis of new DNA in *E. coli*. Data presented represent an initial inoculum of 2200 CFU per well and a culture period of 11 hours. The growth suppressive effect on *E. coli* is what would be expected if the phenomena demonstrated herein were due to a receptor-mediated mechanism.

FIG. **10** delineates the lack of an effect of selected adrenergic receptor agonists upon this process. In this particular graph, the α-receptor agonist octopamine, and the dual α- and β-receptor agonist ephedrine were found to have no effect on Gram-negative growth. This particular graph presents a 10 hour culture of *E. coli* having an initial inoculum of 25 CFU per well. These findings are significant, since a growth promoting effect similar to norepinephrine would be anticipated if the underlying mechanism of this effect was any of the known α- or β-receptors.

FIG. **11** depicts the effect of active and inactive enantiomers of the β-receptor agonist isoproterenol. Although the exact mechanism of this effect has not yet been elucidated, it appears that the modality of the effect of catecholamines in these microorganisms is receptor-modulated. Thus, it would be anticipated that isoproterenol would have an effect similar to the data of FIG. **10**, i.e. no effect. Conversely, at very high concentration there is, in fact, an extremely significant effect. For this reason, both the active (−) and the inactive (+) enantiomers were tested. The data presented are for a 10-hour culture of *E. coli* with an initial inoculum of 25 CFU per well. The fact that both enantiomers produced such a strong effect at high concentration leads to the conclusion that this occurrence is neither receptor specific, nor involves the commonly known β-receptors. Although all aspects have not been fully elucidated as of yet, it is presently believed that a previously unknown third type of receptor is responsible for the catecholamine-induced DNA synthesis observed. These data indicate that some type of positive interaction with this heretofore unknown receptor is

being induced by such a high concentration of agonist. Similar results are also obtained with antagonists, and both sets of data appear to fall within the well-recognized non-specific effects on receptors at very high concentrations. Although not included in figure form herein, a similar effect was not obtained for other Gram-negative bacteria. For example, an essentially flat curve was obtained when these studies were repeated with *Y. enterocolitica*.

Consideration of the data presented to this point confirms what is already well known in the fields of clinical microbiology and public health, that *E. coli* holds a vast capacity for adaptation in nature. It is ubiquitous and in an infectious state, possession of a neurotransmitter receptor would greatly enhance its capacity to utilize the bodily mechanism of releasing catecholamines into the blood stream during stress to its own advantage. The data in FIG. 11 is significant in that it illustrates an ability to also utilize related compounds when such compounds are available at high concentration levels.

FIG. 12 demonstrates the lack of effect of the α-adrenergic receptor antagonist benextramine on the growth of a 20 hour culture of *E. coli* with an initial inoculum of 30 CFU per well. Referring to the bar graph designated "A", the effect of norepinephrine on *E. coli* growth is compared to control medium (CON). As illustrated in the graph labeled B, norepinephrine enhanced growth continues as anticipated irregardless of benextramine concentration. Note that the final concentration of norepinephrine is the same (5×10^{-5}M).

FIG. 13 depicts the effect of the β-adrenergic receptor antagonist alprenolol on a 20 hour culture of *E. coli* with an initial inoculum of 30 CFU per well. Again, norepinephrine stimulates growth in comparison to control and the presence of alprenolol has no inhibiting effect on norepinephrine enhanced growth, as depicted in graph "B". Graph A demonstrates norepinephrine-induced growth in the absence of alprenolol.

FIG. 14 demonstrates the effect of a dual α- and β-receptor agonist, ephedrine, on a 20 hour culture of *Staphyloccus aureus* with an initial inoculum of 27 CFU per well. The growth of this Gram-positive bacterium is not decreased in the presence of this agonist as it is in the presence of norepinephrine. In light of these results, the α-receptor agonist, octopamine, was tested and found to produce some inhibition of growth. This is somewhat analogous to the events in FIG. 11 involving active and inactive enantiomers of isoproterenol.

Table 2 demonstrates the effect of norepinephrine and various α- and β-receptor agonists on the growth of *Y. enterocolitica*. Mean DPM values were obtained for quadruplicate cultures of the indicated compounds. The standard error of the mean did not exceed 10%. Concentrations for all compounds are 10^{-4}M. Abbreviations are: 0, control (no compound added); NE, (−)-norepinephrine; OCT, (−)-octopamine; (−)-isoproterenol; (+)-isoproterenol; and (−)-ephedrine. Values shown were obtained at 36 hours. The number of colony forming units for CON indicates the number of bacteria present in each well at initiation of culture. Once again, the significant growth enhancing effect of norepinephrine is evident in view of insignificant effect by related α- and β-agonists.

To summarize the contents of FIGS. 9–14, receptor blockade by known α- and/or β-antagonists neither induced nor inhibited the growth of Gram-negative bacteria. Similarly, the introduction of α- or β-agonists in moderate quantities to cultures of Gram-positive organisms or in small to large

quantities to cultures of Gram-negative organisms had no effect upon growth. A slight decrease in growth was detected when the Gram-positive bacterium *S. aureus* was grown in the presence of large quantities of the α-agonist, octopamine. These data indicate that a receptor-mediated process is involved, but it does not utilize the known α- or β-adrenergic receptors. Although the process has not yet been fully elucidated, it is believed that Gram-negative organisms possess a non-α, non-β receptor with a unique configuration for catecholamines which gives them a unique advantage in overwhelming a host's defensive mechanisms. During sepsis or related conditions, adrenal and sympathetic stimulation increase the level of circulating catecholamines in order to enhance cardiac and metabolic activity in the host. The activity of Gram-negative microorganisms under these conditions does not appear to be a typical dose response. Although not fully elucidated, it presently appears that in addition to possession of a receptor for some or all of these catecholamines, these organisms emit lipopolysaccharides which block the host's α- and β-adrenergic receptors. Thus, they prevent the re-uptake of these circulating compounds by the host to ensure that they will be able to exploit them instead.

To further substantiate the claim that a novel receptor is involved in the augmentation of growth in the presence of catecholamines, studies were repeated with the Gram-negative bacterium *Y. enterocolitica*. Table 2 demonstrates that in this bacterium, the administration of both α- and β-adrenergic receptor agonists produce no effect, yet in *E. coli* they produce enhancement of growth. Thus, *Y. enterocolitica* is more selective in its use of catecholamines. In particular, an initial inoculum of 130 CFU per well was treated with (+)-octopamine (an α-agonist), (−)-isoproterenol (a β-agonist), (+)-isoproterenol (an inactive enantiomer), and (−)-ephedrine (a dual α-, β-agonist). The incubation period was 36 hours.

INDUSTRIAL APPLICATIONS

The heretofore described effect of including catecholamines in basal culture medium for Gram-negative bacteria has important industrial ramifications. For example, the inclusion of norepinephrine in a culture of *E. coli* grown in a lactose broth was found to substantially increase the yield of glucose. FIG. 15 indicates the results of standard commercial glucose assays 24 hours after inoculation of lactose broth with *E. coli*. Thus, on a commercial scale, the proliferation of *E. coli* in the presence of norepinephrine may be exploited to produce increased yields of glucose. Similar processes with slight variation in starting materials, and according to known methods, will increase the yield of other commercially valuable products such as ethanol.

FIG. 6 demonstrates expression of β-galactosidase activity in a culture of *E. coli*. Although total expression of β-galactosidase activity is increased, actual activity per organism is suppressed. This indicates alteration of this operon and activation of genes which allow the protein to be expressed in *E. coli* is modulated in the presence of norepinephrine. Thus, this provides another example of a significant enhancement in the production of commercially viable products which may be attained by the inclusion of catecholamines, such as norepinephrine, in the appropriate culture medium.

Yet another industrial application of the method of the present invention involves clinical diagnosis of bacterial infections. I have found that current culturing techniques sometimes fail to disclose the presence of such infection

when these microbes are in a phase of low concentration. Such a test will be inappropriately designated as negative, despite the presence of a low concentration of infectious agent. Thus, the inclusion of defined quantities of catecholamines in laboratory diagnostic media will augment growth, as demonstrated in FIGS. 3 and 5. This may aid in the recovery, subsequent identification and quantitation of bacterial disease during clinical diagnosis. Consequently, this will also avoid false negatives in clinical reporting.

Similarly, when biomass degradation is desired, bacterial growth could be significantly increased by the appropriate inclusion of catecholamines. In this manner, subsequent usage of the biomass is enhanced.

One skilled in the art will also recognize that the addition of at least one catecholamine to basal culture medium is beneficial to suppress unwanted growth of susceptible organisms in some industrial applications.

In addition to the augmentation of activity of commercially available products, the addition of catecholamines to basal culture medium and further study may lead to the identification of gene regulating mechanisms for these processes. The subsequent engineering of bacteria with enhanced or altered catecholamine gene regulation capabilities has many industrial applications.

FIGS. 17–20 demonstrate that the effect of norepinephrine as described herein is receptor mediated, and that the receptor involved is a new type of receptor that is not one of the presently known receptors, e.g. α_1, α_2, β_1, or β_2. These figures were obtained using widely accepted methods of neurotransmitter receptor analysis, including methods described in *Methods of Neurotransmitter Receptor Analysis*, Yamamura H. L, S. J. Enna, and M. J. Kuhar, Eds., New York: Raven Press, 1990. According to these accepted methods, these figures satisfy the accepted criteria for identification of a novel receptor.

FIG. 17 provides a competition/displacement curve for ^{3}H-norepinephrine, in which the log of the concentration of cold norepinephrine is plotted on the abscissa and the total amount of bound ^{3}H-norepinephrine (DPM) is plotted on the ordinate. Zero concentration for cold norepinephrine is represented by a box, whereas the presence of cold norepinephrine is indicated by circles. The resultant curve demonstrates that once ^{3}H-norepinephrine has been bound to its receptor on *E. coli*, the addition of increasing amounts of cold norepinephrine results in displacement of the ^{3}H-norepinephrine from the receptor. Because displacement is indicated, this plot provides further evidence that the phenomenon is receptor-mediated.

FIG. 18 provides a saturation curve for ^{3}H-norepinephrine. Bound radioligand is presented on the ordinate and free radioligand is presented on the abscissa, as obtained from a mixture of free radioligand and cold norepinephrine. Thus, nonspecific binding (NSB), specific binding (SB), and total binding (TB) are plotted as a function of free radioligand concentration. The curves presented in FIG. 18 are analogous to those predicted for any receptor mediated process, wherein nonspecific binding appears as an essentially straight line, and is essentially parallel to the high concentration region of the curve for total binding of radioligand. Predictably, the specific binding curve tends to saturate (level off) at high radioligand concentration.

FIGS. 19A through 19C provide competition/displacement curves for the binding of *E. Coli*, grown in commercially available nutrient broth. In these curves, known antagonists for the known adrenergic receptors are applied to cultures killed by sodium azide, to investigate if

the binding of catecholamines is through one of the known α_1, α_2, or β-adrenergic receptors. The value for zero concentration of cold norepinephrine is represented by a box, whereas the presence of cold norepinephrine is indicated by circles. Specifically, FIG. **19A** demonstrates that the addition of ^{3}H-prazosin, a known α_1 receptor antagonist, does not result in any binding that can be displaced by the addition of cold norepinephrine. If the receptor was of a known α_1 subtype, then a competition/displacement curve similar to that shown for FIG. **17** would have been obtained. FIG. **19B** demonstrates that the addition of ^{3}H-rauwolscine, a known α_2 receptor antagonist, does not result in any binding that can be displaced by the addition of cold norepinephrine. If the receptor was of a known α_2, subtype, then a competition/displacement curve also similar to that shown for FIG. **17** would have been obtained. FIG. **19C** demonstrates that the addition of ^{3}H-dihydroalprenolol, a known β_1 and β_2 receptor antagonist does not result in any binding that can be displaced by the addition of cold norepinephrine. If the receptor was of a known β subtype, then a competition/displacement curve similar to that shown for FIG. **17** would have been obtained.

In summary, these adrenergic blocking agents are known to effectively bind their respective known receptors, yet the competition/displacement curves did not reveal the presence of any of the known α_1, α_2 or β receptors. When this information is combined with that provided in FIGS. **1** through **18**, it is surprisingly concluded that a previously unknown receptor is mediating this process.

FIG. **20** provides a tissue dependency curve for *E. coli*. As may be predicted for a receptor-mediated process, specific binding (SB) to the novel receptor is proportional to tissue (bacterial) concentration at low levels, and counts of ^{3}H-norepinephrine specifically bound to its receptor on the bacteria plateau at high concentrations of bacteria when high numbers of receptors are present. In this plot of bacteria:ligand concentration dependence, specific binding (SB), as indicated by DPM, is presented on the ordinate and bacterial concentration, in CFU/ml, is presented on the abscissa.

Although FIGS. **17–20** demonstrate the specificity of this novel receptor for the catecholamines, it does not preclude that differences in receptor number (or density), as well as receptor affinity, may differ among various infectious agents. Further, my preliminary findings indicate that the density and affinity of the receptor for a given infectious agent may differ as a function of the medium or nutritive environment in which the infectious agent is grown. For example, culture of the gram-negative bacterium *Escherichia coli* in a serum-containing medium results in a catecholamine receptor density and affinity which is higher than when *E. coli* is cultured in a nutrient broth of standard microbiological medium not containing serum.

To substantiate my assertion that these findings indicate a receptor-mediated process, I conducted further experiments to test stereoselectivity and demonstrate that a nutrient-based theory will not similarly account for the detected augmentation in growth. Tested, pure (+)-norepinephrine was applied to cultures of *Y. enterocolitica* and my results indicate that the nonphysiological enantiomer did not augment growth to the degree that the active enantiomer, (−)-norepinephrine, did.

Because it has, thus, been demonstrated that the norepinephrine-induced augmentation of growth in gram-negative bacteria is based upon binding to a previously unknown receptor, it is useful to develop a method of binding this receptor for the purpose of enhancement or

suppression of bacterial concentrations. In use, when it is desired to augment a population of gram-negative bacteria, the inclusion of a compound known to bind this receptor, such as norepinephrine, will produce the desired result.

The method of affecting the rate of proliferation of living organisms or vectors, such as bacteria, may be effected by the introduction of a compound, such as a known agonist or antagonist, which will specifically bind with the novel receptor demonstrated in FIGS. **17** through **19**. In a living system, an initial assessment of a need to modify the level of presence of a neurotransmitter chemical, such as norepinephrine, is performed. Such an assessment may be made by incubating the organism or vector, as shown at Block **1**, then evaluating the need to modify its population level. as shown at Block **2**. The result of this assessment determines the antimicrobial agent and treatment protocol which will be administered, as at Block **3**. In the clinical setting, this agent will be selected upon proper identification of a pathogen, typically by culturing. In a state of stress, increased levels of norepinephrine are released by a host mammalian organism. Because, as demonstrated herein, certain gram-negative pathogens have been identified as possessing a novel receptor for circulating catecholamines, and because growth is augmented in the presence of these compounds, it is desirable to block this receptor by administration of a suitable agent, since it is not possible, or if possible, not practical, to block the host organism's release of these compounds. Blocking this novel receptor in the presence of an abundance of such compounds ensures that norepinephrine-enhanced growth will be suppressed.

Alternatively, as described in reference to FIGS. **15** and **16**, it is desirable in some applications to increase the population of a microbe such as *E. coli.*, to obtain a by-product of its growth for commercial use. This may be accomplished on an industrial scale by including a catecholamine, such as norepinephrine, in the culture broth and insuring that this compound remains available as the population grows as at Blocks **4** and **5**. For example, populations of *E. coli* grown in a lactose broth feature a substantially increased yield of glucose when norepinephrine is added to the medium. The method involved in obtaining this result includes the steps of determining the quantity of neurotransmitter substance required to produce a threshold effect on the rate of proliferation of the *E. coli*,

adjusting the broth concentration of this neurotransmitter to this level, then assessing the efficacy of the concentration. If the concentration is not optimal, as at 6, then it is adjusted upwards or downwards until the desired enhancement in glucose yield is obtained, as at 7, as a result of the stimulating effect of the presence of the neurotransmitter. The product may then be harvested, as at Block **8**.

As described herein, the living organisms or vectors which possess the genetic complement enabling possession of the novel receptor described herein include vertebrates, invertebrates, unicellular animals, multicellular animals, living tissue, unicellular plants, multicellular plants, and phages. Although described in reference to gram negative bacteria, this method has particular applicability to the control of proliferation of all infectious agents, including mycobacteria and viruses.

Finally, it has been documented that certain plants contain large amounts of catecholamines. Since it has also been documented that the presence or absence of certain bacteria may repress or enhance growth processes in these plants, it is industrially significant that these bacteria thrive or are repressed in the presence of catecholamines.

This invention has been described herein in considerable detail in order to comply with the Patent Statutes and to provide those skilled in the art with the information needed to apply the novel principles and to construct and use such specialized components as are required. However, it is to be understood that the invention can be carried out by specifically different equipment and devices and that various modifications, both as to equipment details and operating procedures, can be accomplished without departing from the scope of the invention itself.

What is claimed is:

1. A method of suppressing the growth of Gram-positive bacteria in a host medium, said host medium being selected from the group consisting of in vitro and cell cultures, said method comprising the introduction of an effective amount of a catecholamine to the host medium to act directly on the growth of Gram-positive bacteria.

2. A method as claimed in claim **1** wherein the catecholamine is selected from epinephrine and norepinephrine.

* * * * *

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[11] **Patent Number:** 5,629,349

[45] **Date of Patent:** May 13, 1997

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Attorney, Agent, or Firm—Haugen and Nikolai, P.A.

[57] **ABSTRACT**

A method for modulating both in vivo and/or in vitro bacterial growth by administration of neurochemicals is disclosed. This method involves the recognition of a novel receptor for these compounds and includes the steps of: assessing need to apply a neurotransmitter chemical based upon the nature of the living organism, determining an amount of neurotransmitter chemical required to produce an effect upon the rate of proliferation, applying the neurotransmitter chemical to the living organism, assessing efficacy of this application in reducing or enhancing the rate, and repeating these steps intermittently to monitor the actual rate of proliferation, whether accelerated or depressed. A method is further disclosed wherein the subject neurotransmitter chemical, such as a catecholamine, is added to a basal culture medium for the purpose of augmenting (or suppressing) growth. This step is useful for the commercial production of organisms such as bacteria. It is further useful for the increased production of commercially useful byproducts of this augmented growth, such as glucose or ethanol.

2 Claims, 16 Drawing Sheets

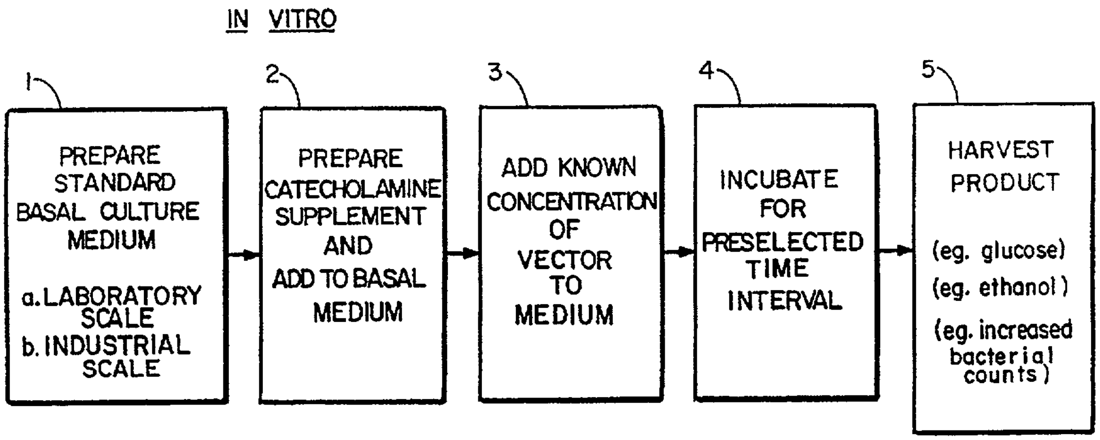

IN VITRO
1
PREPARE STANDARD BASAL CULTURE MEDIUM
a. LABORATORY SCALE
b. INDUSTRIAL SCALE
2
PREPARE CATECHOLAMINE SUPPLEMENT AND ADD TO BASAL MEDIUM
3
ADD KNOWN CONCENTRATION OF VECTOR TO MEDIUM
4
INCUBATE FOR PRESELECTED TIME INTERVAL
5
HARVEST PRODUCT
(eg. glucose)
(eg. ethanol)
(eg. increased bacterial counts)

FIG. 1

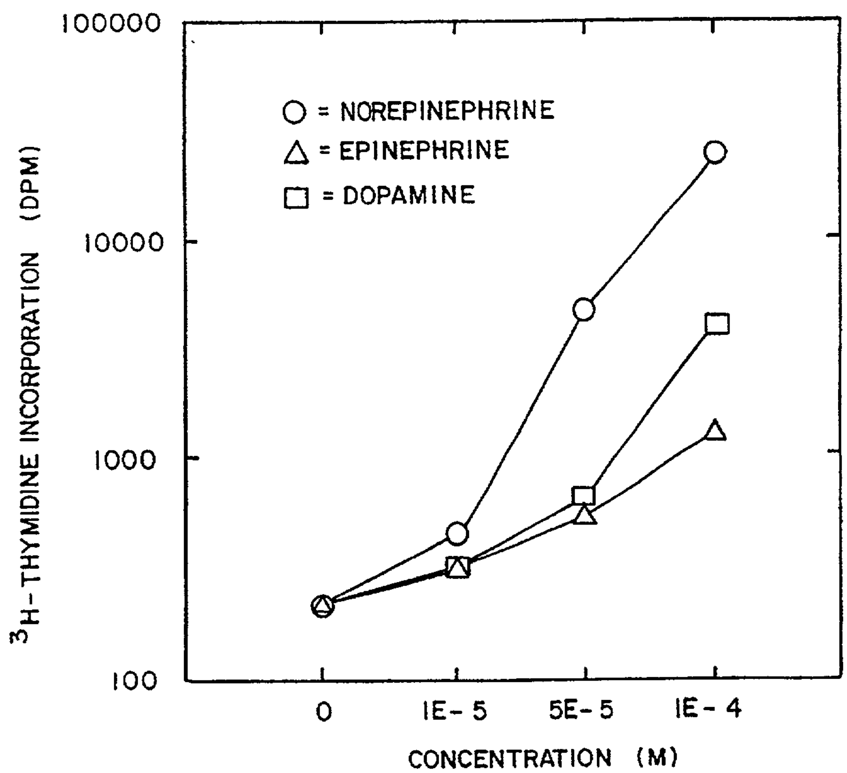

100000
10000
1000
100
○ = NOREPINEPHRINE
△ = EPINEPHRINE
□ = DOPAMINE
^{3}H-THYMIDINE INCORPORATION (DPM)
0
1E-5
5E-5
1E-4
CONCENTRATION (M)

FIG. 3

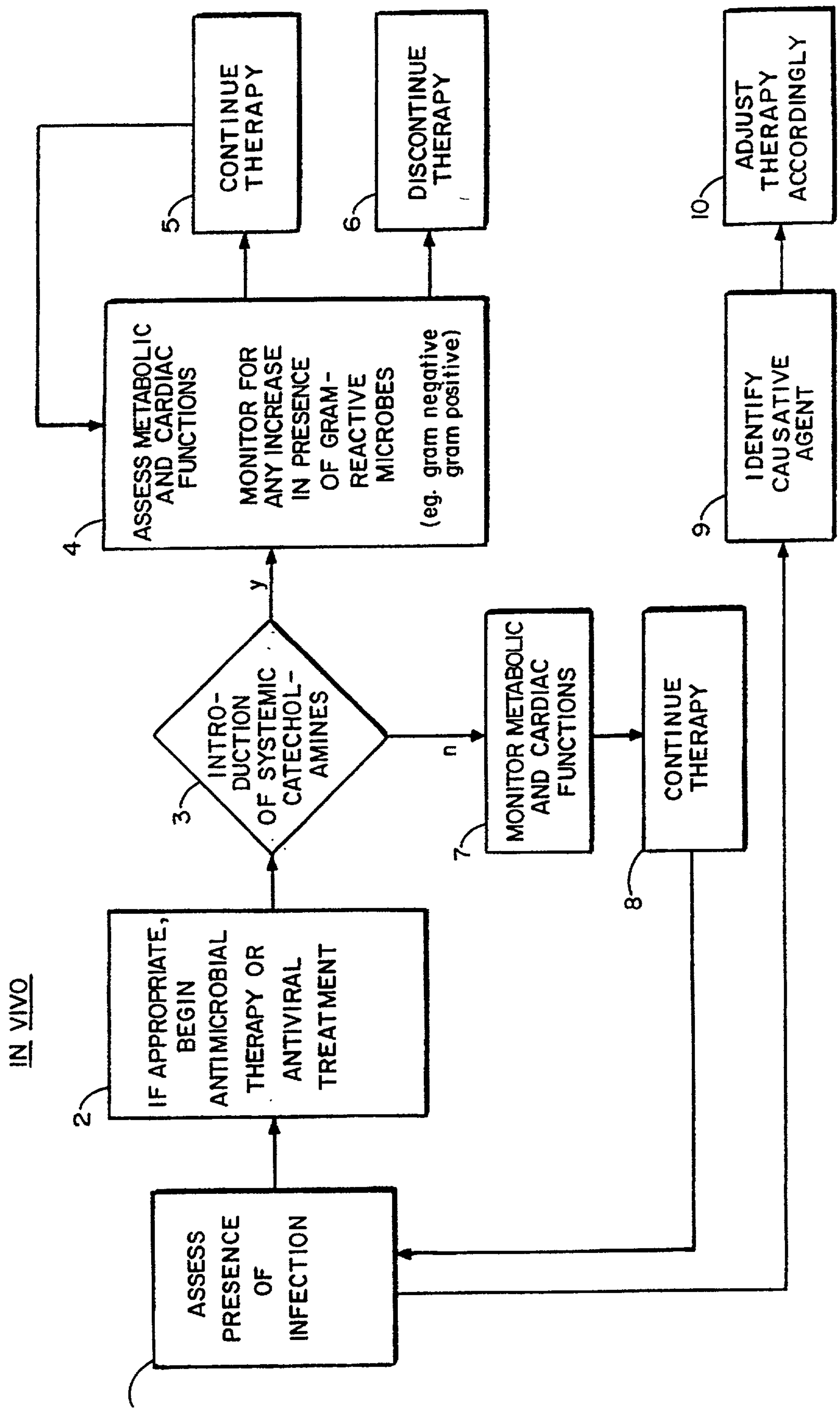
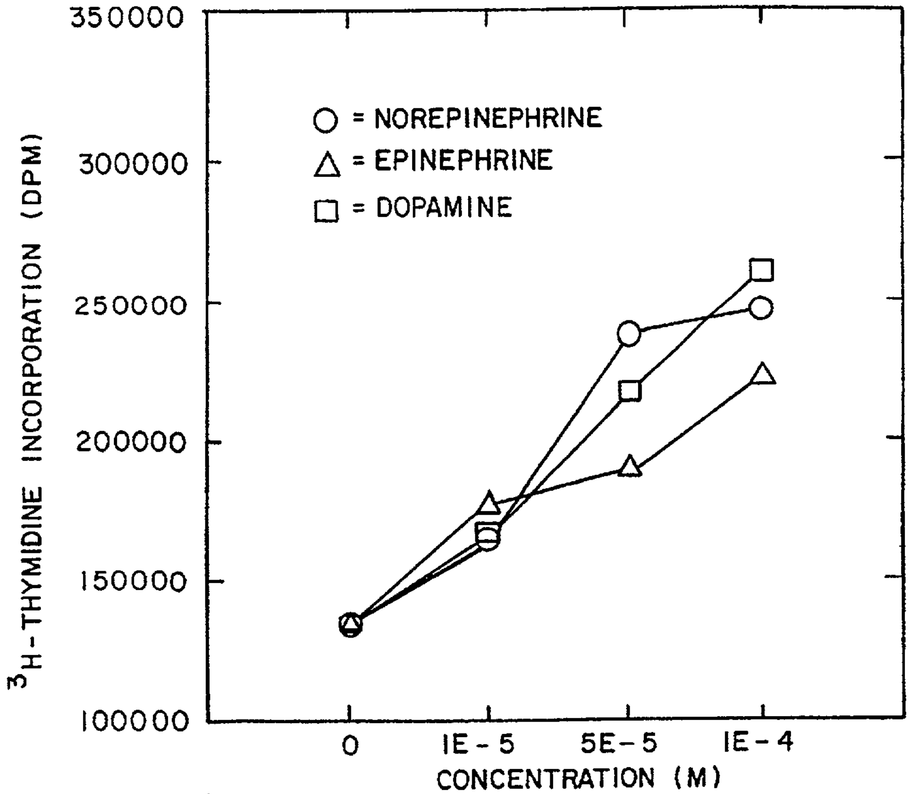
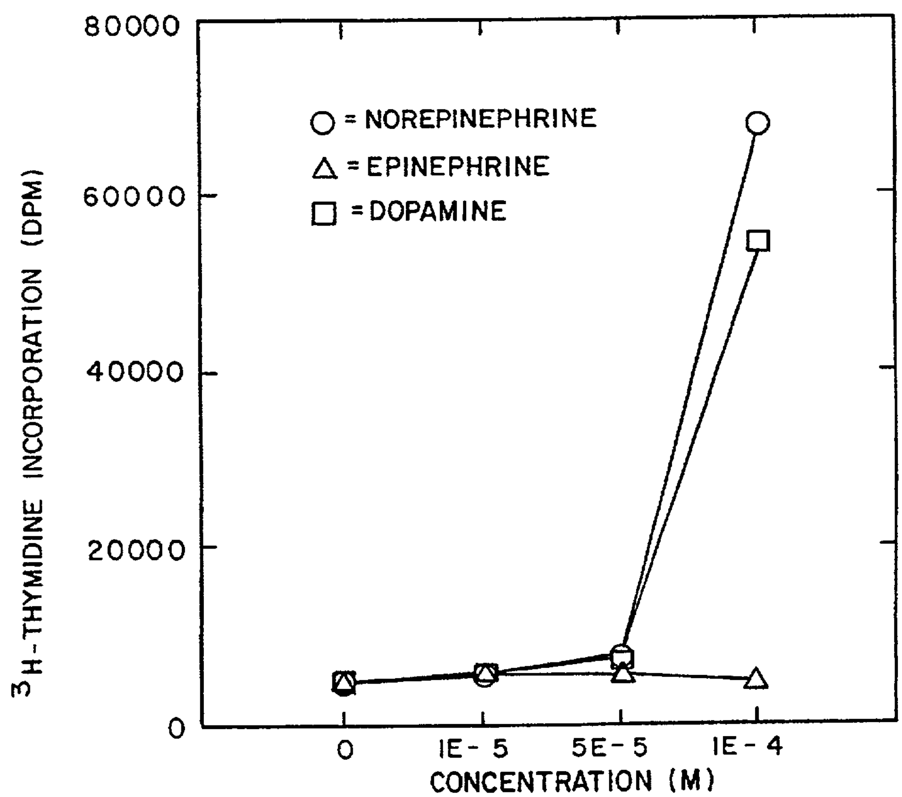

IN VIVO
ASSESS PRESENCE OF INFECTION
1
IF APPROPRIATE, BEGIN ANTIMICROBIAL THERAPY OR ANTIVIRAL TREATMENT
2
INTRO-DUCTION OF SYSTEMIC CATECHOL-AMINES
3
y
n
ASSESS METABOLIC AND CARDIAC FUNCTIONS
MONITOR FOR ANY INCREASE IN PRESENCE OF GRAM-REACTIVE MICROBES
(eg. gram negative gram positive)
4
CONTINUE THERAPY
5
DISCONTINUE THERAPY
6
MONITOR METABOLIC AND CARDIAC FUNCTIONS
7
CONTINUE THERAPY
8
IDENTIFY CAUSATIVE AGENT
9
ADJUST THERAPY ACCORDINGLY
10

FIG. 2



350000
300000
250000
200000
150000
100000
^{3}H-THYMIDINE INCORPORATION (DPM)
○ = NOREPINEPHRINE
△ = EPINEPHRINE
□ = DOPAMINE
0
IE-5
5E-5
IE-4
CONCENTRATION (M)

FIG. 4



80000
60000
40000
20000
0
^{3}H-THYMIDINE INCORPORATION (DPM)
○ = NOREPINEPHRINE
△ = EPINEPHRINE
□ = DOPAMINE
0
IE-5
5E-5
IE-4
CONCENTRATION (M)

FIG. 5

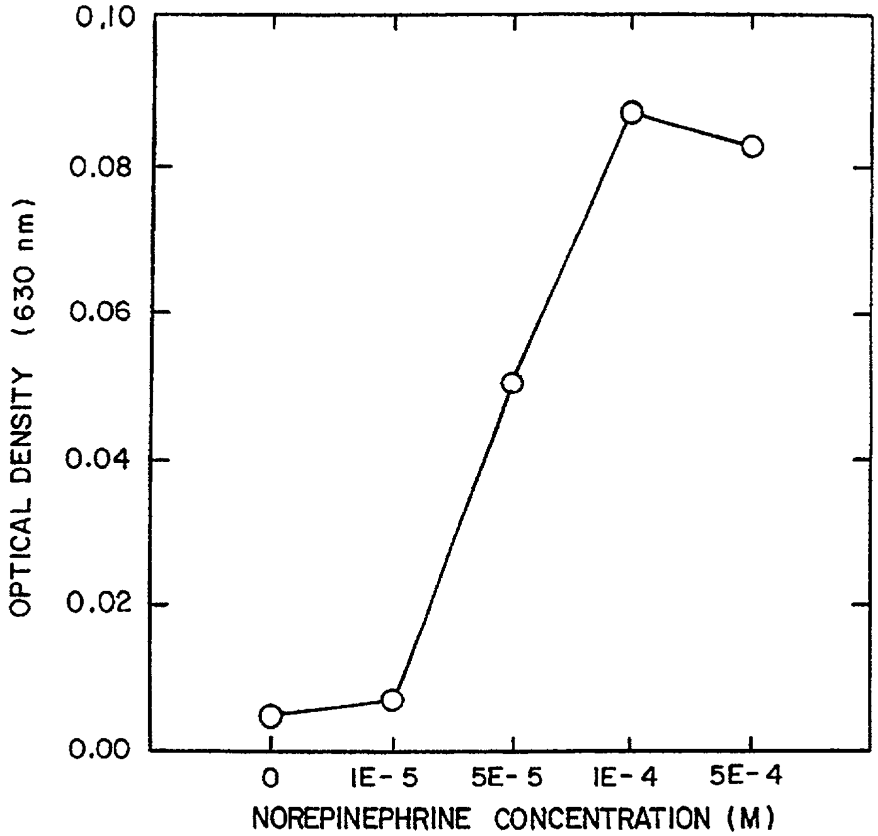

0.10
0.08
0.06
0.04
0.02
0.00
OPTICAL DENSITY (630 nm)
0
IE-5
5E-5
IE-4
5E-4
NOREPINEPHRINE CONCENTRATION (M)

FIG. 6

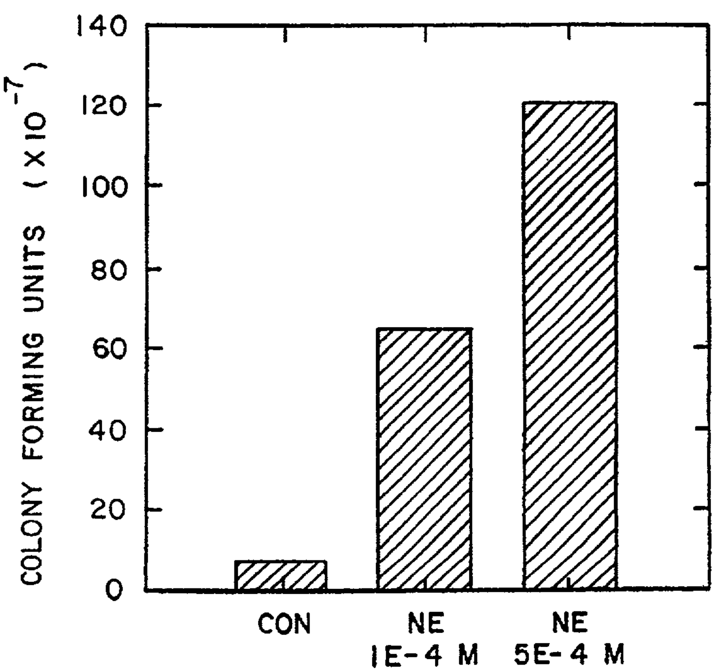

140
120
100
80
60
40
20
0
COLONY FORMING UNITS (X10^{-7})
CON
NE
IE-4 M
NE
5E-4 M

FIG. 7

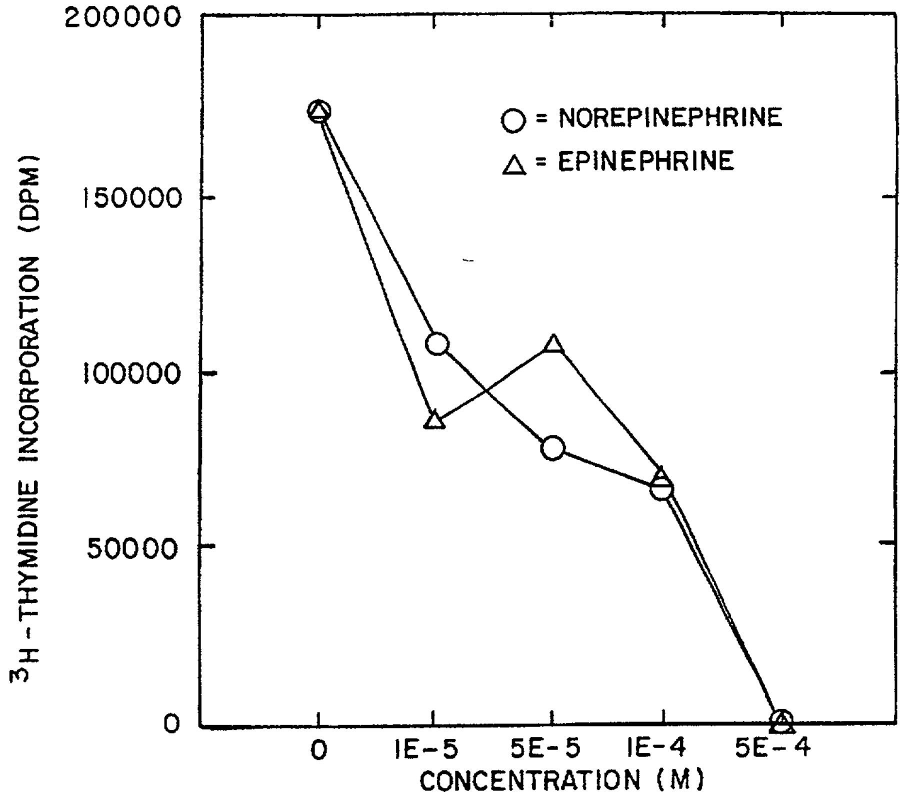

200000
150000
100000
50000
0
^{3}H-THYMIDINE INCORPORATION (DPM)
○ = NOREPINEPHRINE
△ = EPINEPHRINE
0
1E-5
5E-5
1E-4
5E-4
CONCENTRATION (M)

FIG. 8

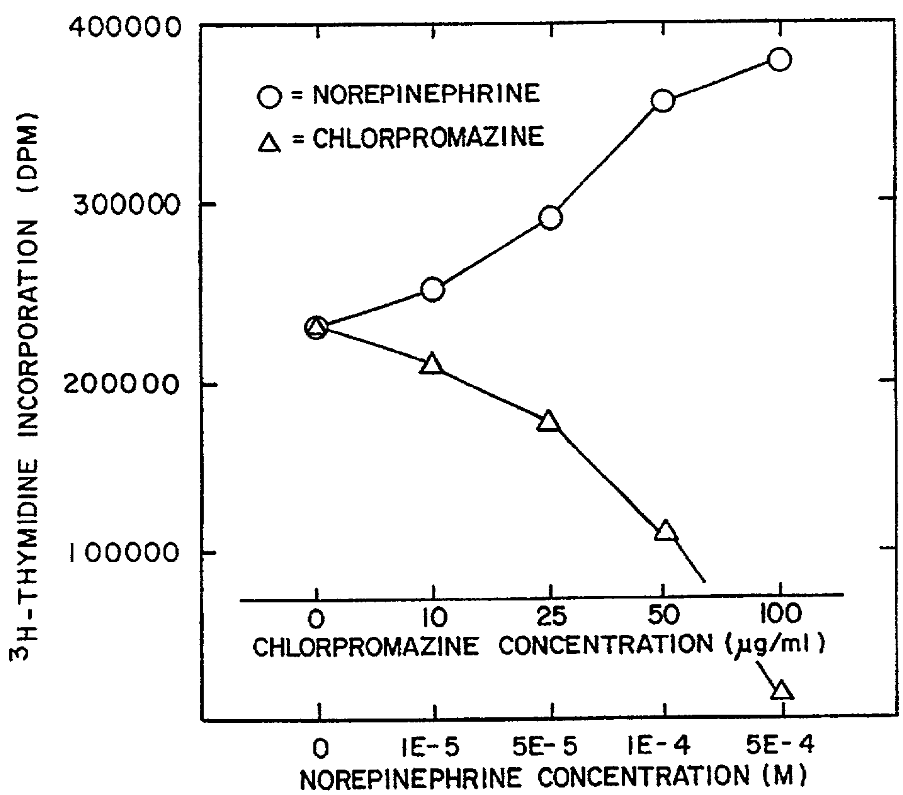

400000
300000
200000
100000
^{3}H-THYMIDINE INCORPORATION (DPM)
○ = NOREPINEPHRINE
△ = CHLORPROMAZINE
0
10
25
50
100
CHLORPROMAZINE CONCENTRATION (μg/ml)
0
1E-5
5E-5
1E-4
5E-4
NOREPINEPHRINE CONCENTRATION (M)

FIG. 9

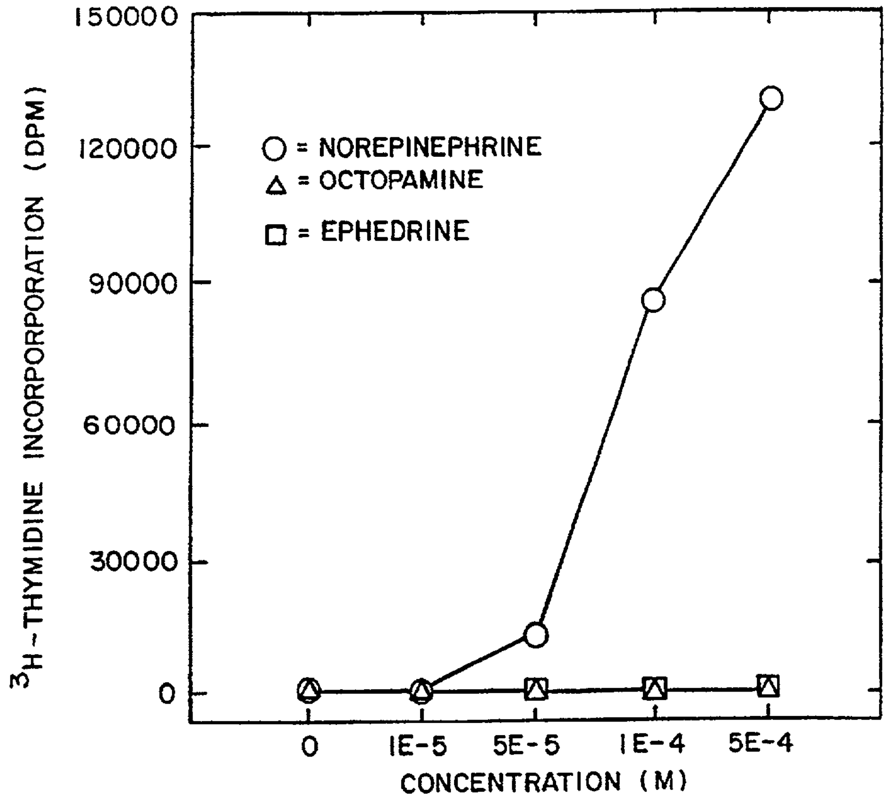

150000
120000
90000
60000
30000
0
^{3}H-THYMIDINE INCORPORATION (DPM)
○ = NOREPINEPHRINE
△ = OCTOPAMINE
□ = EPHEDRINE
0
1E-5
5E-5
1E-4
5E-4
CONCENTRATION (M)

FIG. 10

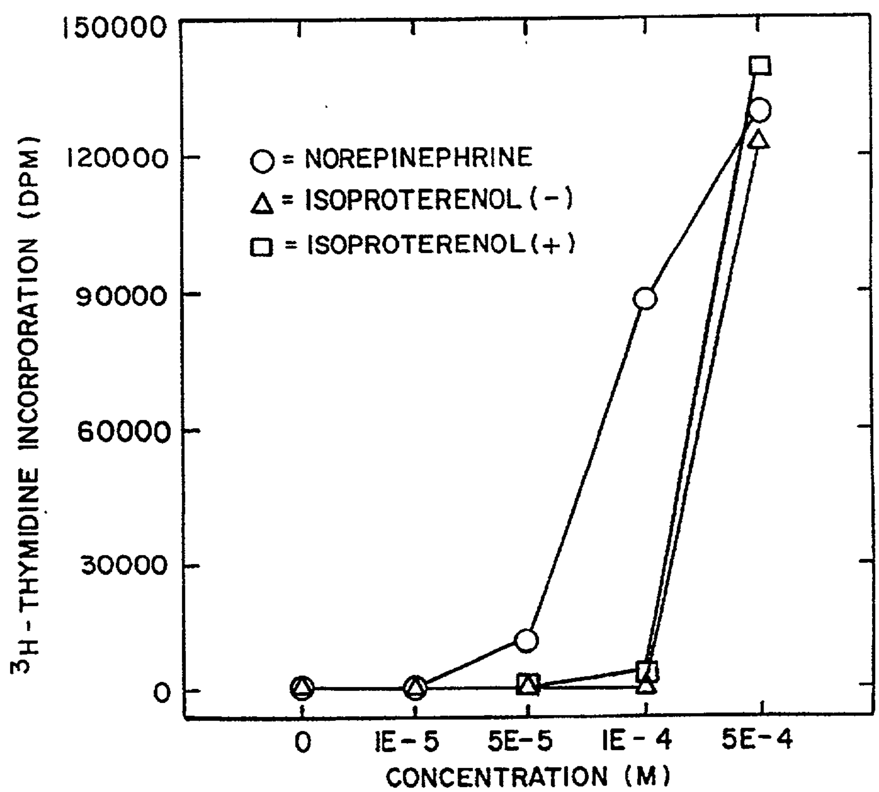

150000
120000
90000
60000
30000
0
^{3}H-THYMIDINE INCORPORATION (DPM)
○ = NOREPINEPHRINE
△ = ISOPROTERENOL (-)
□ = ISOPROTERENOL (+)
0
1E-5
5E-5
1E-4
5E-4
CONCENTRATION (M)

FIG. 11

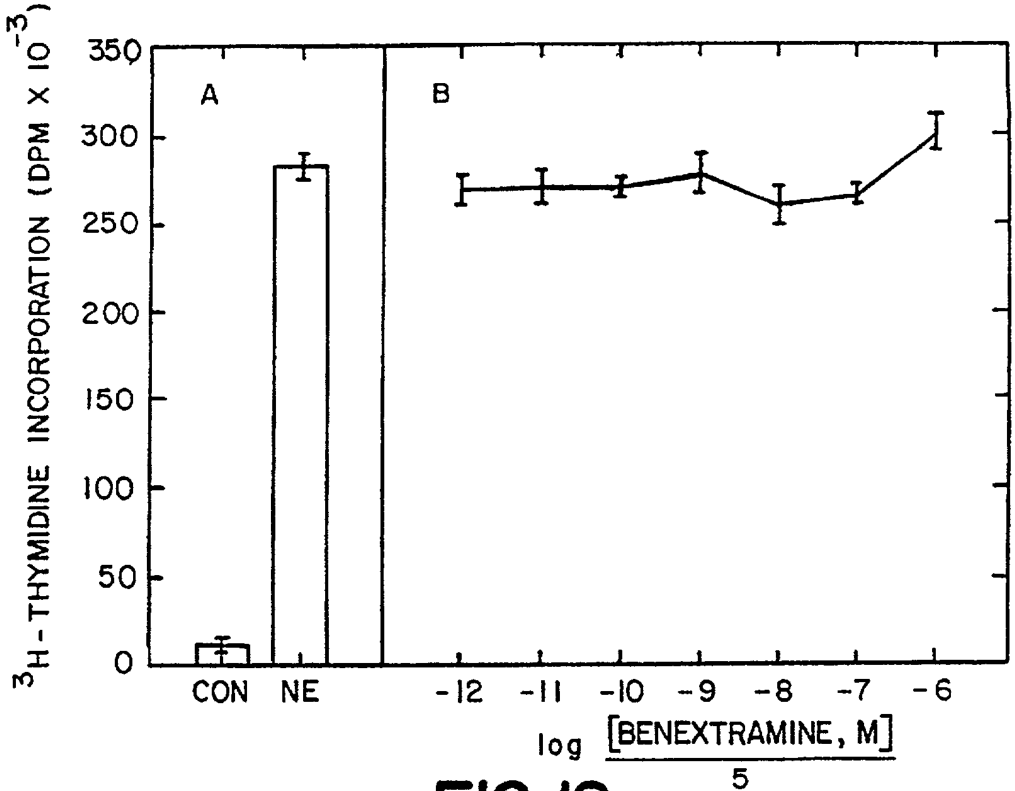

^{3}H-THYMIDINE INCORPORATION (DPM X 10^{-3})
350
300
250
200
150
100
50
0
A
B
CON
NE
-12
-11
-10
-9
-8
-7
-6
log [BENEXTRAMINE, M] / 5

FIG. 12

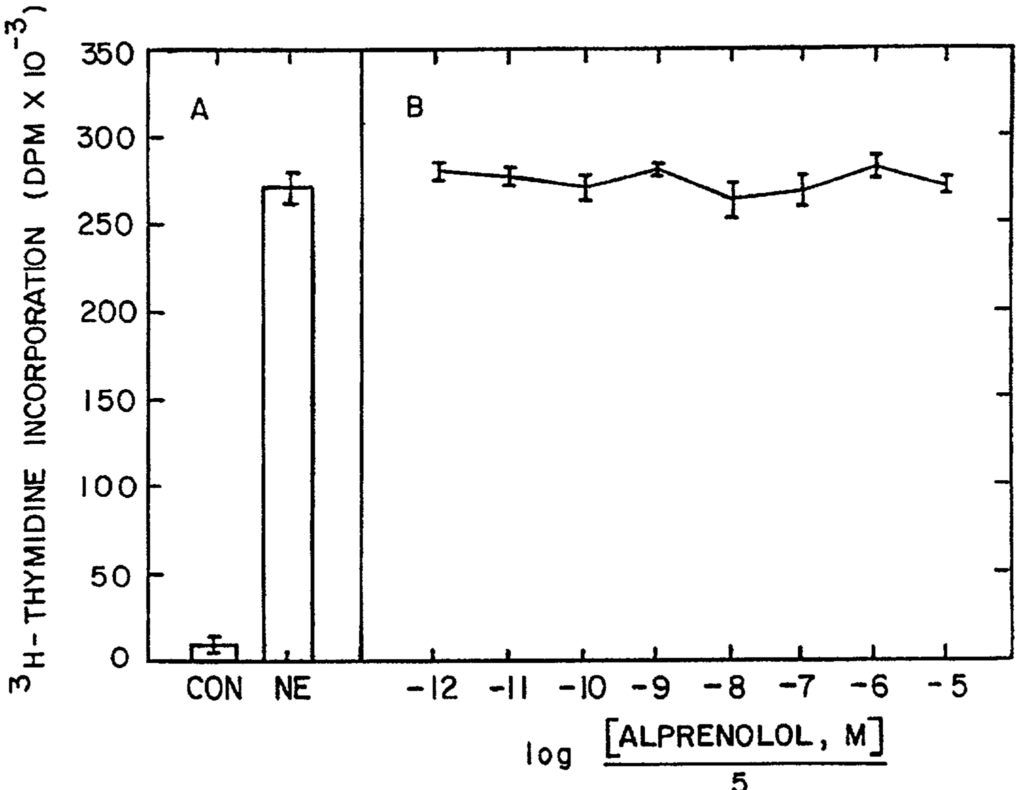

^{3}H-THYMIDINE INCORPORATION (DPM X 10^{-3})
350
300
250
200
150
100
50
0
A
B
CON
NE
-12
-11
-10
-9
-8
-7
-6
-5
log [ALPRENOLOL, M] / 5

FIG. 13

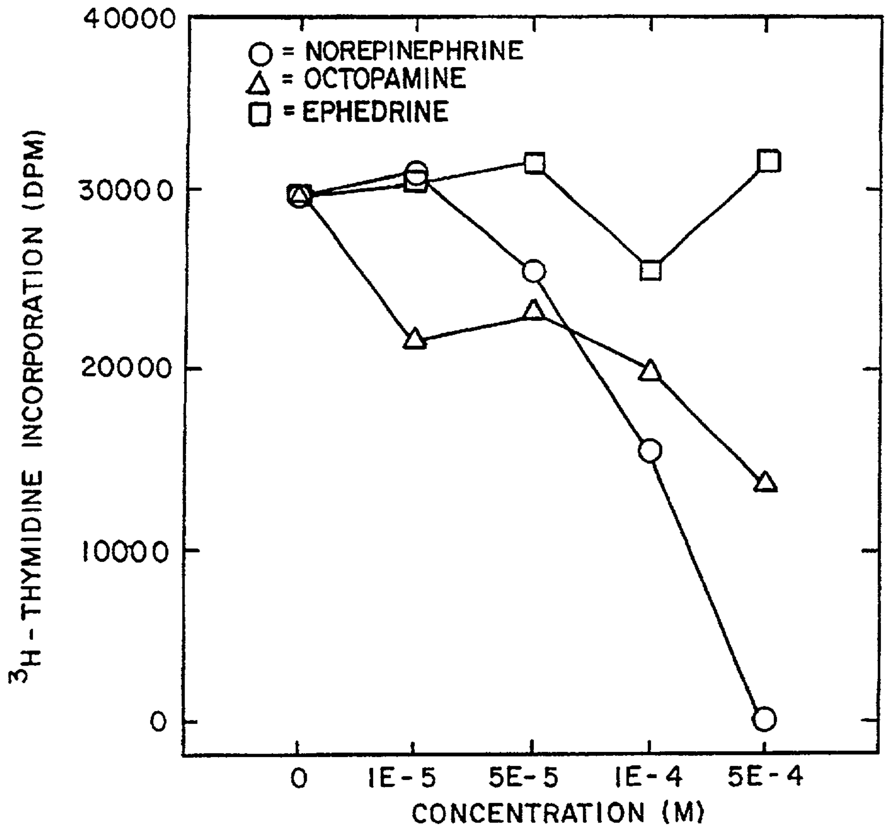


FIG. 14

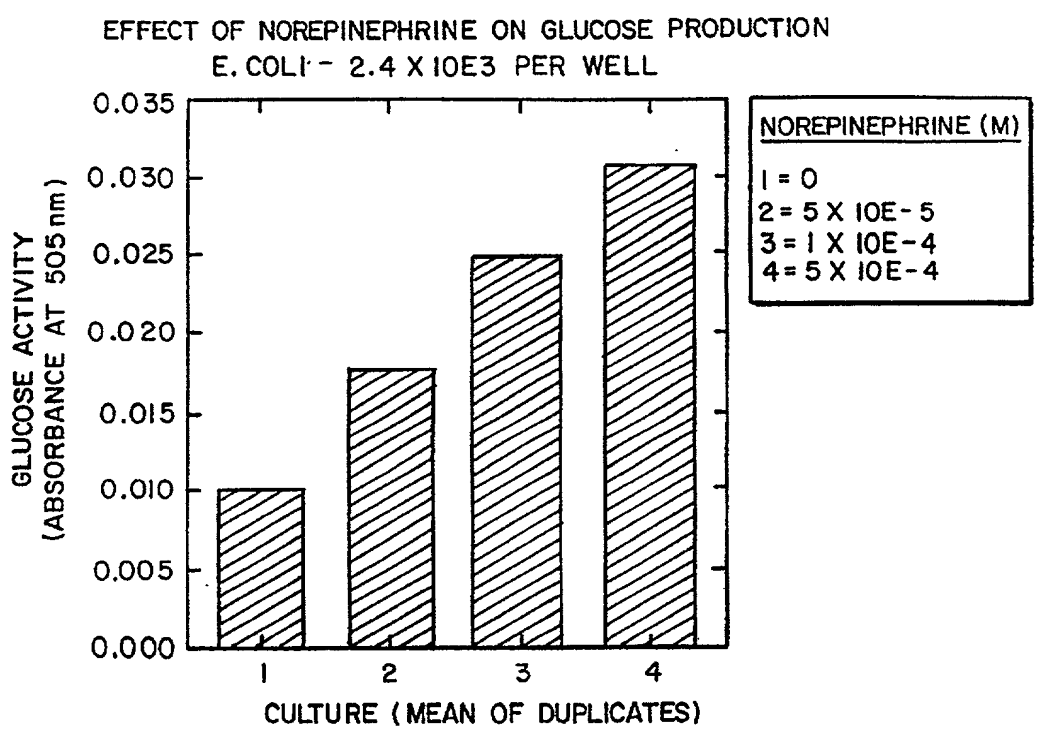


FIG. 15

09020525.07260

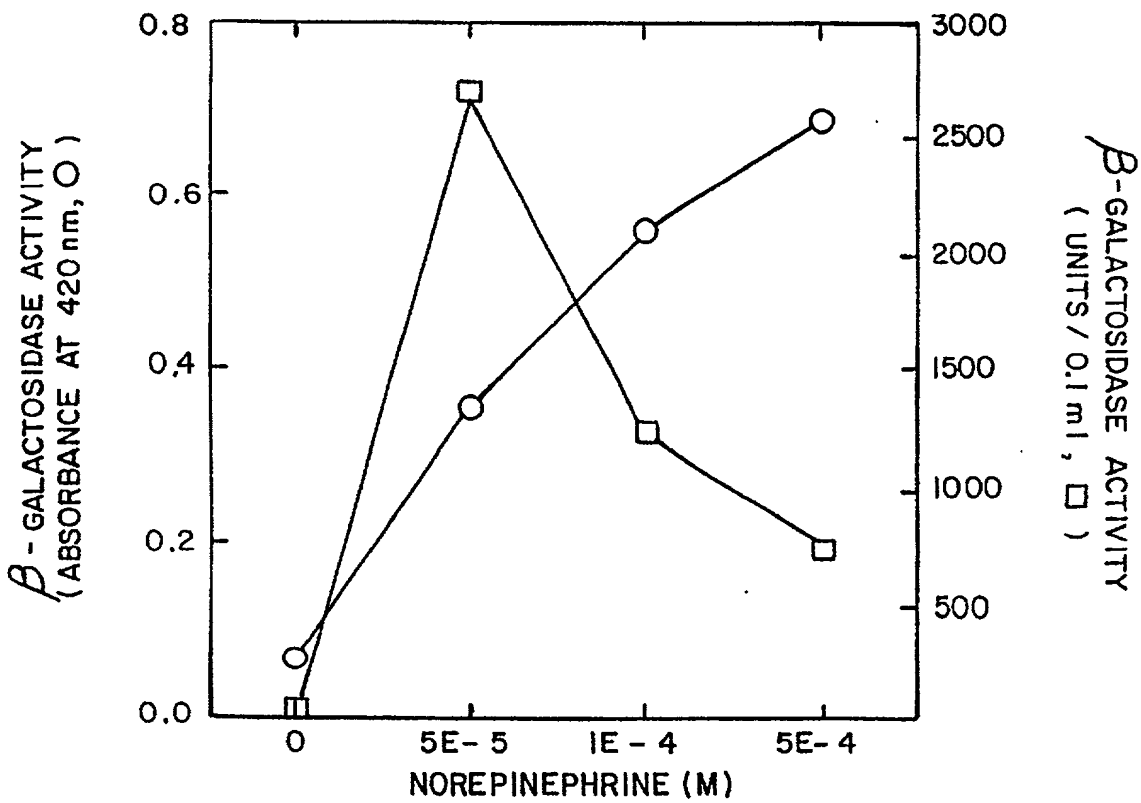
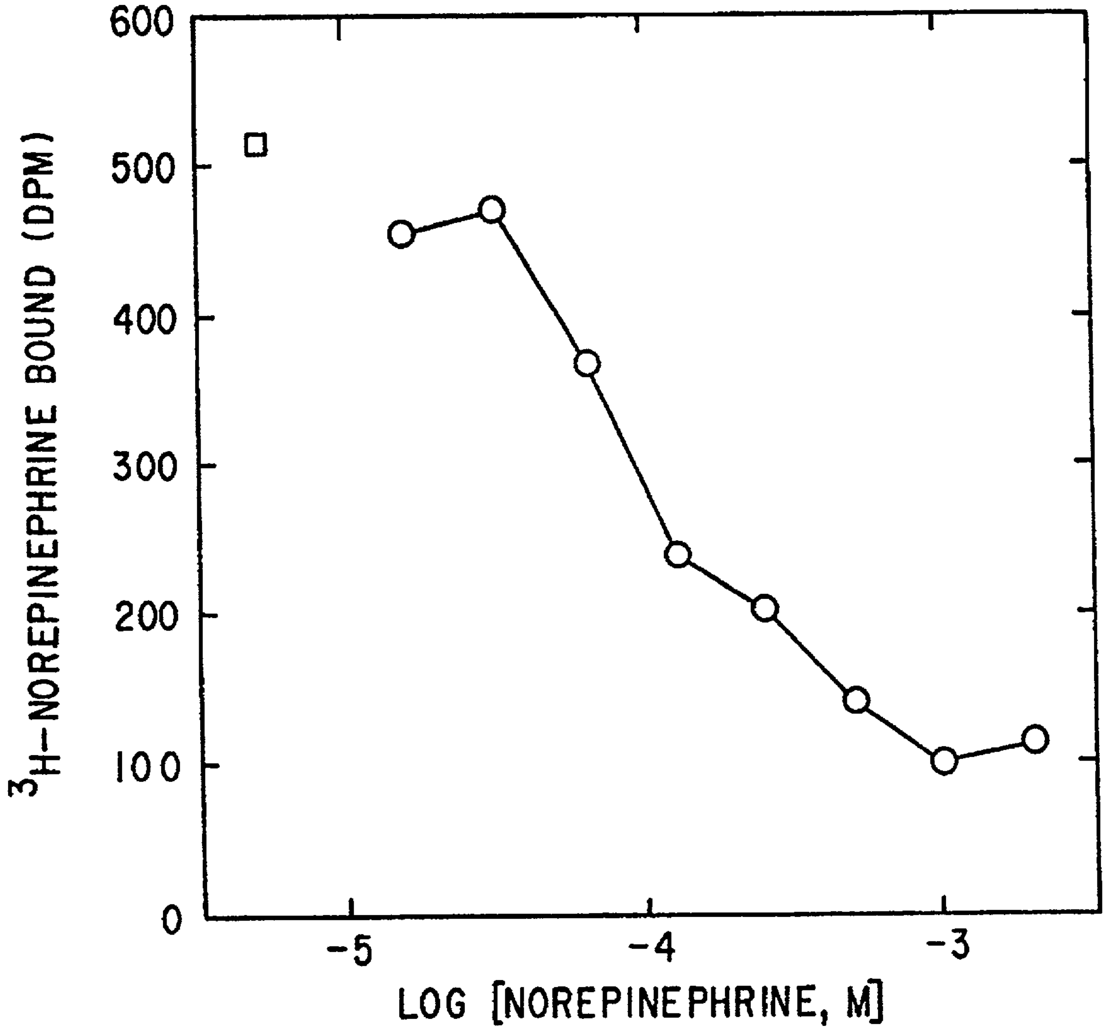

0.8
0.6
0.4
0.2
0.0
3000
2500
2000
1500
1000
500
0
5E- 5
IE - 4
5E- 4
NOREPINEPHRINE (M)
β- GALACTOSIDASE ACTIVITY (ABSORBANCE AT 420 nm, ○)
β-GALACTOSIDASE ACTIVITY (UNITS / 0.1 ml , □)

FIG. 16



600
500
400
300
200
100
0
^{3}H-NOREPINEPHRINE BOUND (DPM)
-5
-4
-3
LOG [NOREPINEPHRINE, M]

FIG. 17

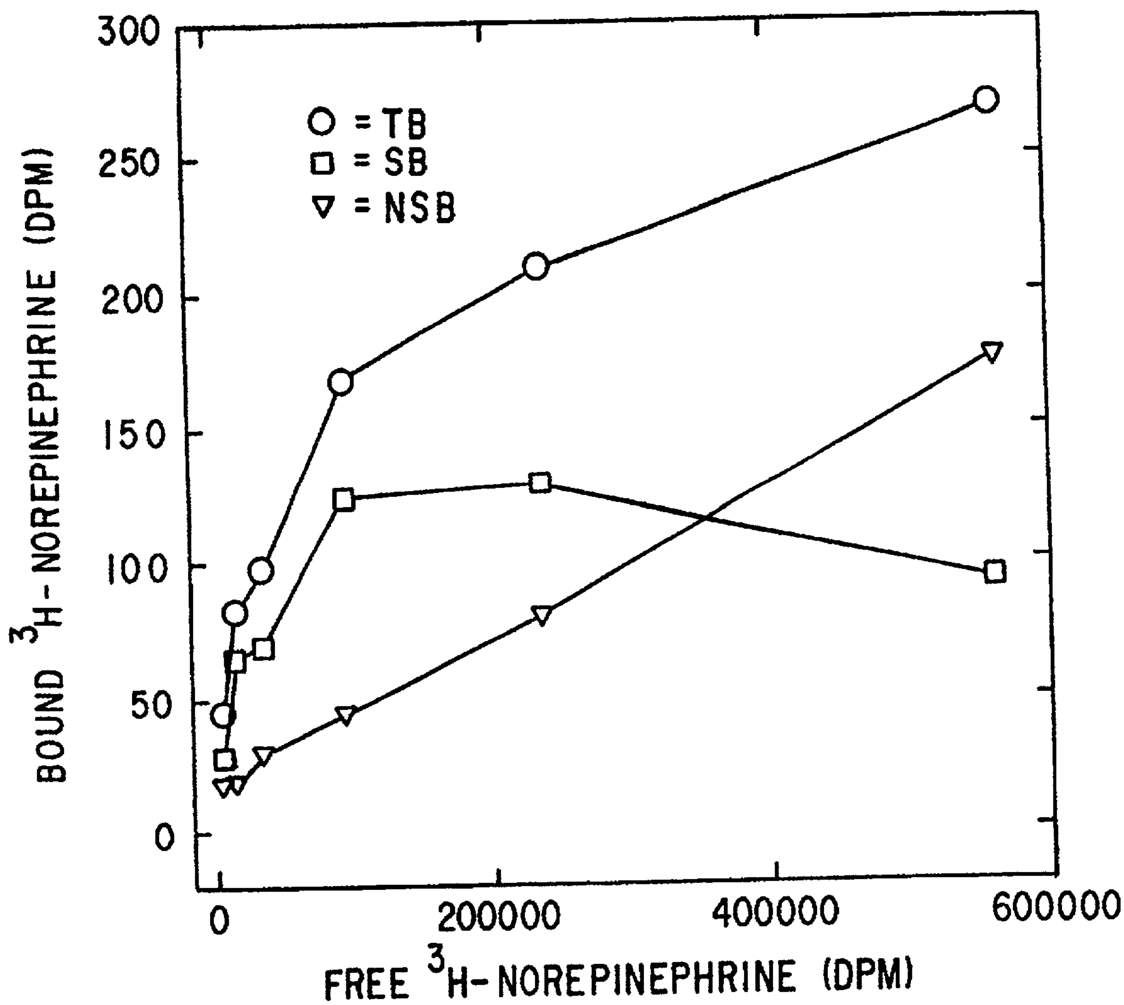

○ = TB
□ = SB
▽ = NSB
BOUND ^{3}H-NOREPINEPHRINE (DPM)
300
250
200
150
100
50
0
0
200000
400000
600000
FREE ^{3}H-NOREPINEPHRINE (DPM)

FIG. 18

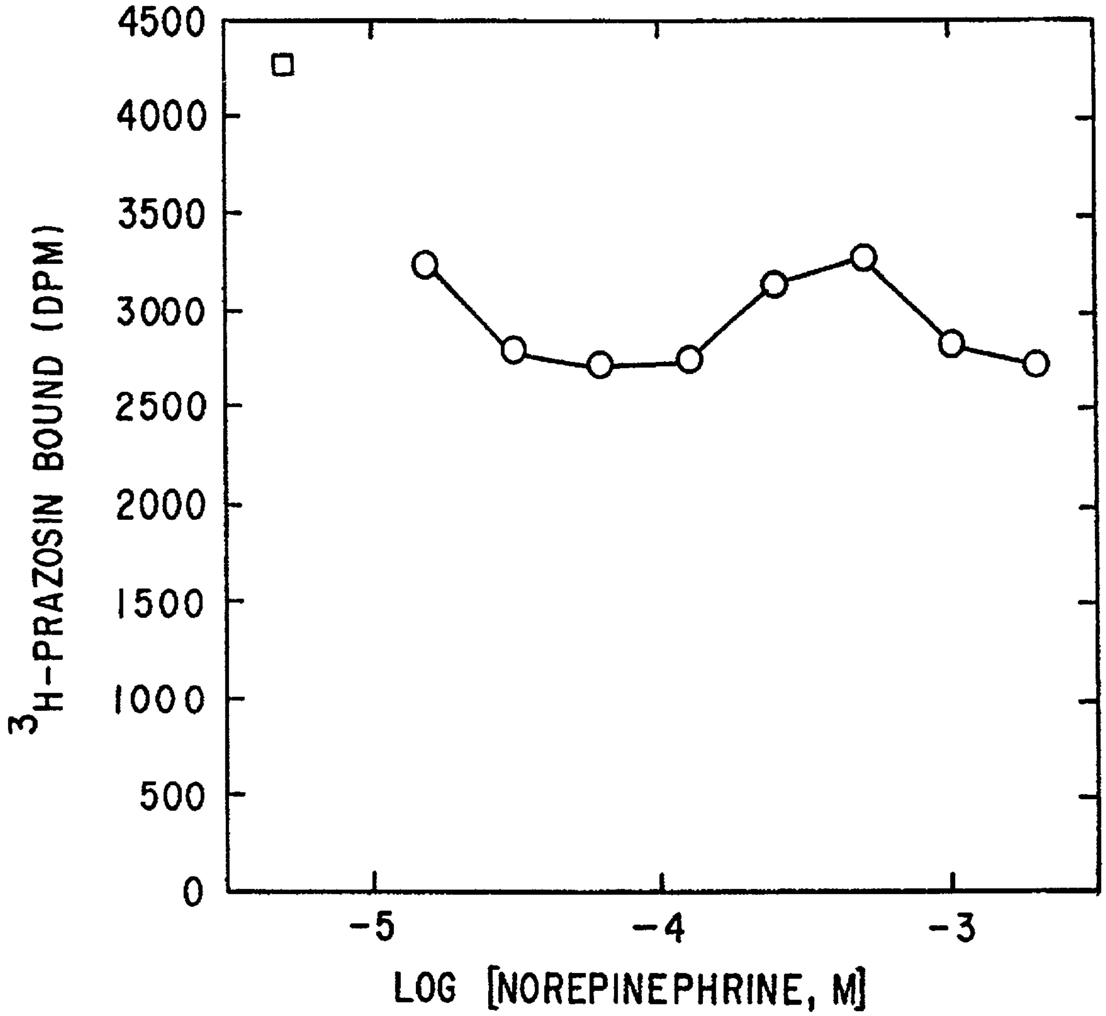


FIG. 19A

09/020,528.56

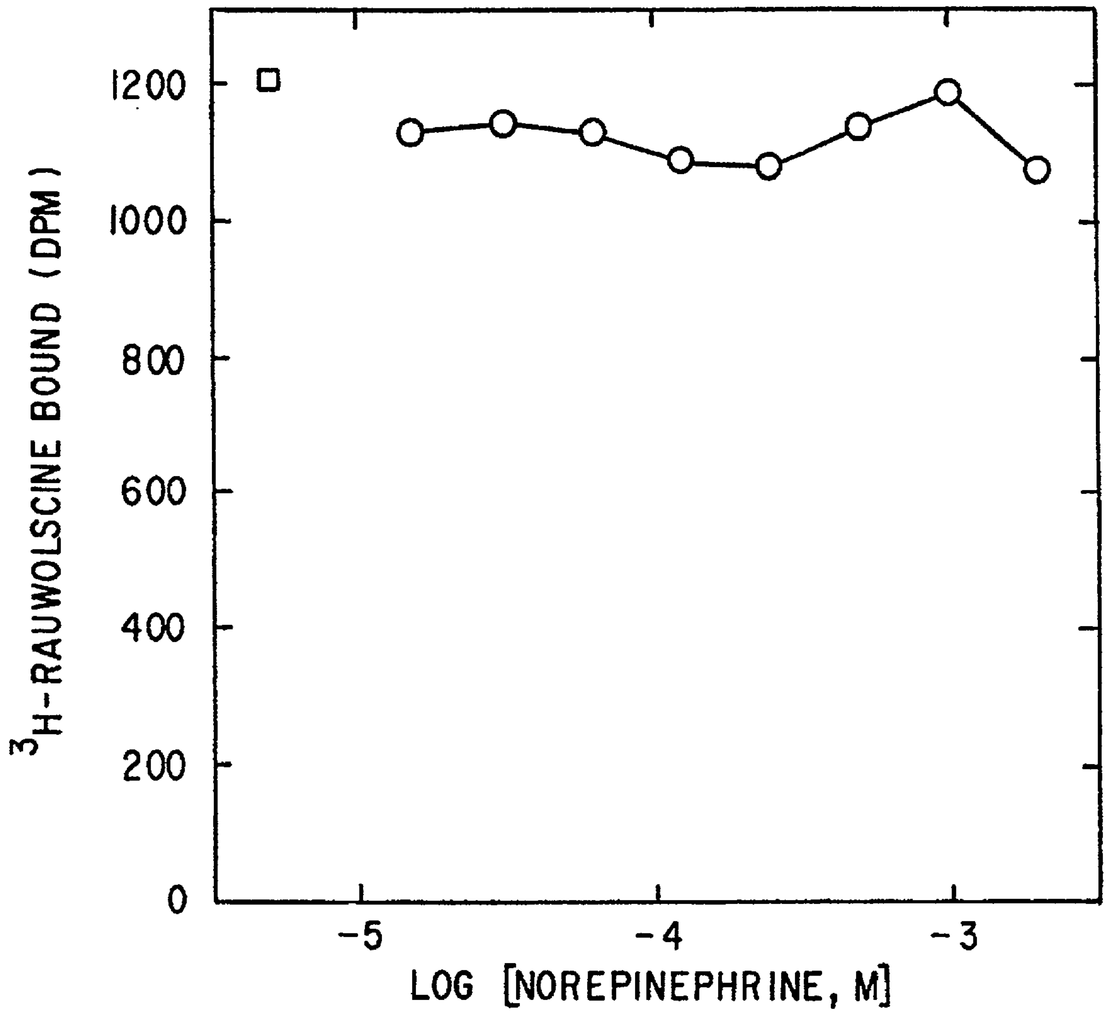


FIG. 19B

09530254.062097

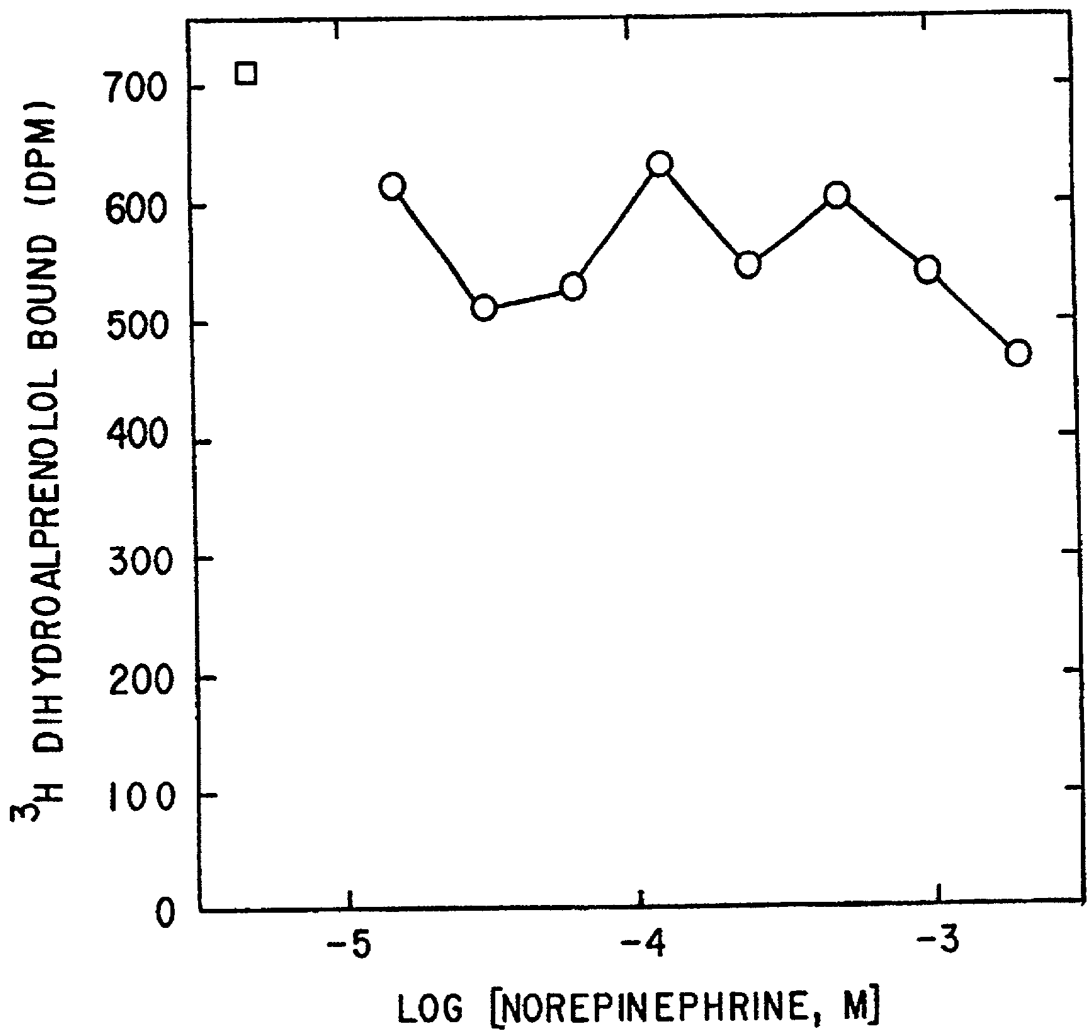

^{3}H DIHYDROALPRENOLOL BOUND (DPM)
700
600
500
400
300
200
100
0
-5
-4
-3
LOG [NOREPINEPHRINE, M]

FIG. 19C

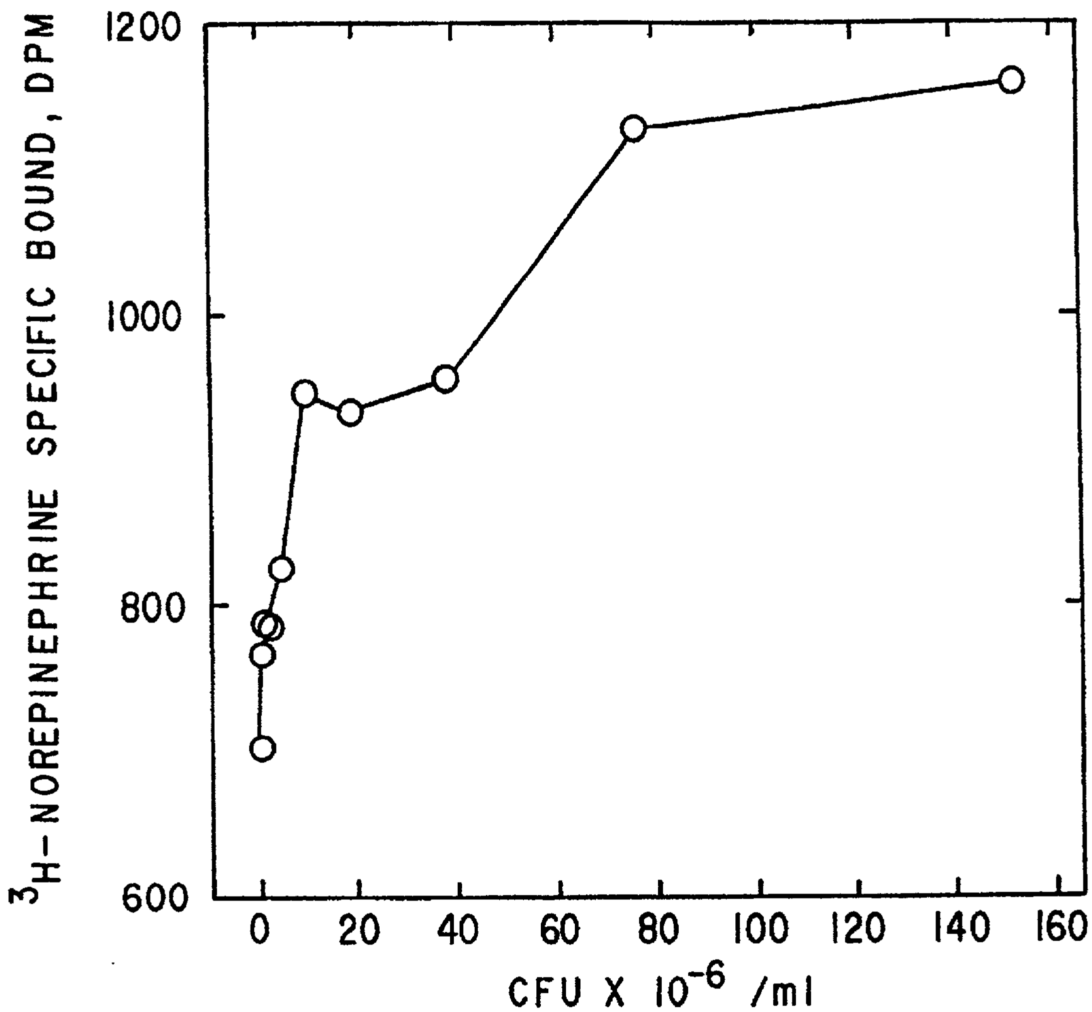

1200
1000
800
600
^{3}H-NOREPINEPHRINE SPECIFIC BOUND, DPM
0
20
40
60
80
100
120
140
160
CFU X 10^{-6} /ml

FIG. 20

TABLE 1

MICROBE	CFU	CATECHOLAMINE (DPM)					
		0	NE	EPI	DOPA	MHPG	NOR
YERSINIA ENTERO-COLITICA	80	722	54342	856	6623	853	866
ESCHERICHIA COLI	15	258	24629	1563	4354	1105	632
ESCHERICHIA COLI	1500	135349	243531	226365	255121	183496	95081

FIG. 21

TABLE 2

CFU	COMPOUND (DPM)					
	0	NE	OCI	(-)ISO	(+)ISO	EPH
130	150	29243	159	146	183	297

FIG. 22

08521355.020497

Patent and Trademark Office in connection therewith, and to receive the Letters Patent. Please direct all communications to the following:

Mark A. Litman, Esq.
SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.
1600 TCF Tower
121 South Eighth Street
Minneapolis, MN 55402
(612) 373-6975

CERTIFICATE UNDER 37 CFR §3.73(b)

BioNutrix, LLC, hereby certifies that it is the assignee of the entire right, title, and interest in U.S. Patent No. 5,629,349 identified above by virtue of a contribution agreement from the inventor, Mark Lyte, dated June 17, 1998, a copy of which is submitted concurrently herewith for recordation. To the best of my knowledge and belief, title is in the assignee, BioNutrix, LLC.

Pursuant to 37 C.F.R. §3.73(b) I hereby declare that I am empowered to sign this certificate on behalf of the assignee, BioNutrix, LLC.

I hereby declare that all statement made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true.

Date: 2.1.99

Signature: Ted Schwarzrock
Name : Ted Schwarzrock
Title : President, BioNutrix, LLC

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